SERUM SOLUBLE α KLOTHO AND FGF 23 LEVELS IN CHRONIC KIDNEY DISEASE

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BONAFIDE CERTIFICATE

This is to certify that this dissertation work entitled **"SERUM SOLUBLE α KLOTHO AND FGF 23 LEVELS IN CHRONIC KIDNEY DISEASE"** is the original bonafide work done by **Dr. D. KALPANA**, post graduate student, Institute of Biochemistry, Madras Medical College, Chennai, under our direct supervision and guidance.

Prof. Dr.R.Chitraa, MD., (Guide)
Professor
Institute of Biochemistry
Madras Medical College
Chennai. 600 003.

Prof. Dr. K.Ramadevi. MD.,PhD
Director & Professor,
Institute of Biochemistry
Madras Medical College
Chennai-600 003.

Dean
Madras Medical College and
Rajiv Gandhi Government General Hospital,
Chennai - 600 003.

DECLARATION

I, **Dr.D.KALPANA**, post graduate, Institute of Biochemistry, Madras Medical College, solemnly declare that the dissertation titled **"SERUM SOLUBLE α KLOTHO AND FGF 23 LEVELS IN CHRONIC KIDNEY DISEASE"** is the bonafide work done by me at the Institute of Biochemistry, Madras Medical College, under the expert guidance and supervision of **Professor Dr.R.CHITRAA,** M.D., Institute of Biochemistry, Madras Medical College. The dissertation is submitted to the Tamil Nadu Dr. M.G.R Medical University towards partial fulfillment of requirement for the award of M.D., Degree (Branch XIII) in Biochemistry.

Place: Chennai

Date: **Dr. D.KALPANA**

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CONTENTS

ABBREVIATIONS

1.	CKD	– chronic kidney disease
2.	FGF 23	– fibroblast growth factor
3.	GFR	– glomerular filteration rate
4.	GN	– glomerulonephritis
5.	ANCA	– anti neutrophil cytoplasmic antibody
6.	COL4A5	– gene encoding alpha 5 chain of type V basement membrane collagen
7.	FSGS	– focal segmental glomerulosclerosis
8.	MHC	– major histocompatibility complex
9.	TGF-β	– transforming growth factor β
10.	CTGF	– connective tissue growth factor
11.	ECM	– extra cellular matrix
12.	PDGF	– platelet derived growth factor
13.	CSGF	– colony stimulating growth factor
14.	NADP+	– nicotinamide adenine dinucleotide phosphate
15.	EMT	– epithelial mesenchymal transition
16.	CD	– cluster of differentiation
17.	CCR5	– chemokine receptor 5
18.	CxCR3	– chemokine receptor
19.	EGF	– epidermal growth factor
20.	ET-1	– endothelin 1
21.	HIF	– hypoxia inducible factor
22.	MCP 1	– monocyte chemoattractant protein 1
23.	RANTES	– regulated on activation normal T cell expressed and secreted

24. MDRD – modification of diet in renal disease
25. CKD EPI – chronic kidney disease epidemiology collaboration
26. KL – klotho
27. PTH – parathyroid hormone
28. NaPi–2a – sodium phosphate co transporter
29. TRPV 5 – transient receptor potential vanilloid channel
30. ROMK – renal outer medullarypotassium
31. ADAM – A disintegrin and metalloproteinase
32. RAA – renin angiotensin aldosterone system
33. IL – interleukin
34. Smad II – small worm phenotype and mothers against decapentaplegic
35. Pit 1 and Pit 2 – type III sodium dependent phosphate transporters
36. FGFR 1 – fibroblast growth factor receptor
37. GALNT 3 – polypeptide N – acetyl galactosaminyltransferase 3
38. HS – heparansulphate
39. BaF3 – murine interleukin 3 dependent pro B cell line
40. MAPK – mitogen activated protein kinase
41. HEK 293 – human embryonic kidney cells
42. ESRD – end stage renal disease
43. TNAP – tissue non-specific alkaline phosphatase
44. FEP – fractional excretion of phosphate
45. ERK – extracellular signal regulated kinase
46. NF-κ – nuclear factor kappa

Introduction

INTRODUCTION

Chronic kidney disease is an emerging public health problem with global prevalence between 11 to 13%. There is a paucity of sensitive biomarkers to diagnose early CKD and there are few specific and effective approaches to retard CKD progression and prevention of extra-renal complications.

Pathological variations of Klotho / FGF 23 in chronic kidney disease are implicated as clinical biomarkers and may bring forth novel therapeutic strategies to reduce the cardiovascular risk and slow down the progression of damage to kidney. Chronic kidney disease – mineral and bone disorder causes abnormalities in bone and mineral metabolism or extra skeletal calcification[(1)]. The FGF 23 / Klotho endocrine axis plays a vital role in mineral metabolism. Serum FGF 23 level increases early in chronic kidney disease before the increase of serum phosphate or parathyroid hormone. In CKD early onset of Klotho deficiency contributes to renal FGF 23 resistance and a maladaptive increase in circulating FGF 23. FGF 23 is an early marker of renal injury, increased levels of which predicts adverse clinical outcomes in particular cardiovascular disease. α Klotho functions as an obligatory co-receptor for FGF 23 along with FGF receptor to transduce FGF 23 signaling to regulate calcium and phosphate metabolism as a calico-phosphotrophic hormone.

Review of Literature

REVIEW OF LITERATURE

CKD definition

"CKD is defined as abnormalities of kidney structure or function, present for more than 3 months, with implications for health"[(2)].

Criteria for CKD (either of the following present for more than 3 months)

Markers of kidney damage

Albuminuria

Urine sediment abnormalities

Electrolyte and other abnormalities due to tubular pathology

Abnormalities detected by histology

Structural abnormalities identified by imaging

History of transplantation of kidney

Decreased GFR

GFR < 60 mL/min/1.73m^2 (GFR categories G3a-G5)[(3)].

Classification of CKD based on presence or absence of systemic disease and location within the kidney of pathologic anatomic findings

Systemic diseases affecting the kidney		Primary kidney disease
Glomerular diseases	Diabetes, systemic autoimmune disease, systemic infections, drugs, neoplasia (including amyloidosis)	Diffuse, focal or crescentic proliferative GN; focal and segmental glomerulosclerosis, membranous nephropathy, minimal change disease
Tubulointerstitial diseases	Systemic infections, autoimmune, sarcoidosis, drugs, urate, environmental toxins	Urinary tract infections, stones, obstruction
Vascular diseases	Atherosclerosis, hypertension, ischemia, systemic vasculitis, thrombotic microangiopathy, systemic sclerosis	ANCA-associated renal limited vasculitis, fibromuscular dysplasia
Cystic and congenital diseases	Polycystic kidney disease, Alport syndrome, Fabry disease	Renal dysplasia, medullarycystic disease,podocytopathies

GFR categories in CKD

GFR Category	GFR ($mL/min/1.73m^2$)	Terms
G1	>90	Normal or high
G2	60–89	Mildly decreased
G3a	45–59	Mildly to moderately decreased
G3b	30–44	Moderately to severely decreased
G4	15–29	Severely decreased
G5	< 15	Kidney failure

Pathophysiology of kidney disease

The pathophysiology of CKD is discussed along with renal anatomical and physiological characteristics, in addition to the mechanisms of renal tissue injury and repair.

1. The rate of renal blood flow is roughly about 400mL/100g of tissue per minute, which is much beyond that observed in other well perfused organs such as heart, liver and brain. Consequently, renal tissue is exposed to a significant quantity of potentially noxious circulating agents or substances[(4)].

2. Glomerular filtration depends on high intra and trans glomerular pressure (even in stable physiologic conditions), making the glomerular capillaries more prone to hemodynamic injury, contrary to other capillary beds. Glomerular hypertension and hyperfiltration are the main reasons for chronic renal disease progression.

3. Glomerular filtration membrane has negatively charged molecules at the surface, which acts as a barrier to anionic molecules. When this electrostatic barrier is damaged as a consequence of glomerular injury, plasma proteins will enter the filtrate.

4. The sequential arrangement of nephrons microvasculature (glomerular convolute and the network of peritubular capillaries) and the subsequent position of tubule in regard to glomeruli not only sustain the glomerulo-

tubular balance but also promote the spread of glomerular injury to the tubulointerstitial compartment, exposing tubular epithelium to abnormal ultra-filtrate. The peritubular vasculature underlies glomerular circulation, and glomerular inflammatory mediators following injury may overflow into the peritubular circulation contributing to the subsequent interstitial inflammatory reaction in glomerular disease.

Any reduction in pre-glomerular or glomerular perfusion leads to reduction in peritubular blood flow which, depending on the extent of hypoxia, evokes tubulointerstitial injury and tissue remodeling.

5. The glomerulus should be viewed as a functional unit with each of its constituents (endothelial, mesangial, visceral and parietal epithelial cells – podocytes and extracellular matrix) performing an integral part of normal function. Damaging one will affect the other through various mechanisms, direct cell – cell interaction(e.g., gap junctions), soluble mediators such as chemokines, cytokines, growth factors and changes in matrix composition and basement membrane[(4)].

Major causes of renal injury are

1. Immunologic reactions (initiated by immune cells or immune complexes)
2. Tissue hypoxia and ischemia
3. Exogenous agents like drugs
4. Endogenous substances like glucose or para-proteins and others
5. Genetic defects

Regardless of the cause, glomerulosclerosis and tubulointerstitial fibrosis are main features of CKD.

The pathophysiology of CKD involves specific consideration to mechanisms of glomerular, tubular and vascular injury.

MECHANISMS OF GLOMERULAR IMPAIRMENT

Hereditary defects

It contributes to minor proportion of CKD. Classical example is Alport syndrome, which involves mutation in the COL4A5 gene that encodes the α5 chain of type4 collagen. As a consequence, glomerular basement membrane is irregular with longitudinal layering, splitting or thickening and the patient develops progressive glomerulosclerosis and renal failure. Other causes include thin membrane syndrome, nail–patella syndrome, partial lipodystrophy and familial lecithin–cholesterol acyl transferase deficiency.

Acquired glomerular disease

It is triggered by immune mediated injury, and metabolic and mechanical stress. From the pathologic point of view, it can be broadly divided into three groups

1. Non-proliferative
2. Proliferative
3. Heterogeneous

1. Non-proliferative (without cell proliferation) glomerular disease without glomerular inflammation and without deposition of immunoglobulin[minimal change disease, idiopathic focal, and segmental glomerulosclerosis (FSGS) or with deposition of immunoglobulin, but without glomerular inflammation, most likely because of subepithelial localization of immunoglobulin e.g., membranous nephropathy]

2. Proliferative glomerular disease with deposition of immunoglobulins leading to increased cellularity e.g., lupus nephritis, IgA nephropathy, anti –glomerular basement membrane, post infectious GN), or with severe glomerular injury and inflammation, but without deposition of immunoglobulin (e.g., pauci-immune glomerulonephritis)

3. Heterogenous group of glomerular diseases in systemic diseases like diabetes, amyloidosis and paraproteinemia.

Tissue injury after immune complex deposition is mediated through complement activation resulting in the formation of C5-9 membrane attack complex which appears to be the major effector of glomerular injury through release of chemo tactic C5a and C3a. C5-9-activated cells release chemokines and oxidant proteases, and upregulate adhesion molecules.

T- cells also mediate golomerular injury by the production of nephritogenic antibodies, especially in pauci-immune GN. They interact through their surface receptor /CD3 complex with antigens presented in the clefts of MHC molecules of

endothelial, mesangial and epithelial glomerular cells. Once activated T-cells release cytokines and other inflammatory mediators it causes cytotoxicity and fibrogenesis.

TGF-β and connective tissue growth factor (CTGF) are important in glomerular fibrogenesis as they stimulate production of extracellular matrix (ECM) a key event in the progression of kidney disease, inhibiting tissue proteases.

Glomerular inflammation can either completely recover or resolve with a variable degree of fibrosis. The resolution process requires

- Cessation of further antibody production and immune complex formation
- Degradation and removal of deposited and circulating immune complexes.
- Cessation of recruitment and clearing, of inflammatory cells
- Dispersing of inflammatory mediators
- Normalization of endothelial adhesiveness, permeability and vascular tone
- Clearance of proliferating residual glomerular cells

NON-IMMUNOLOGIC GLOMERULAR INJURY

Hemodynamic, metabolic and toxic injuries can induce glomerular impairment alone or in combination with immunological processes.

SYSTEMIC HYPERTENSION

The kidney is normally protected from systemic hypertension by autoregulation, which can be overwhelmed by high blood pressure, systemic

hypertension translated directly to glomerular filtration barrier causing glomerular injury. Chronic hypertension will lead to arteriolar vasoconstriction and sclerosis with consequent secondary sclerosis and glomerular and tubulointerstitial atrophy. Different growth factors like angiotensin II, EGF, PDGF, CSGF, TGF –β CYTOKINE, activation of stretch activated ion channels and early response genes, are involved in coupling high blood pressure to myo-intimal proliferation and vessel wall sclerosis.

GLOMERULAR HYPERTENSION

It is normally an adaptive response mechanism in remaining nephrons to increased workload resulting from loss of nephrons, whatever may be the cause. This sustained intraglomerular hypertension increases the mesangial matrix production and leads to glomerulosclerosis by ECM accumulation.

Metabolic injury:

Three mechanisms have been postulated to explain the effect of hyperglycemia in tissue damage.

1) Advanced glycated end products:

Glucotoxicity leads to the formation of advanced glycation end products by non enzymatic glycosylation of intracellular proteins where glucose & other glycating compounds attach to amino group and other molecule such as nucleic acid without the aid of enzyme, producing reversible early glycated end products (Schiff's base) and later, irreversible (Amadori products).

These products are slowly converted into advanced glycated end products.

It accumulates in tissue (arterial wall and glomerular basement membrane) by cross linking with the collagen causing atherosclerosis and glomerular dysfunction.

It alters the signal transduction by altering the level of cytokines, hormones and free radicals to contribute the renal and micro vascular complications.

2) Protein kinase C pathway:

Hyperglycemia activates protein kinase c by de novo formation of diacyl glycerol and oxidative stress, leading to secretion of vasodilatory prostanoids, TGF-β1 which contributes to glomerular hyper filtration and mesangial cell expansion.

3) Polyol pathway:

When intracellular glucose concentration is raised, glucose metabolism takes place via sorbitol pathway by the enzyme aldose reductase. It is present in mesangial cells, distal tubular cell, and glomerular epithelial cell of normal kidney. It generates sorbitol in response to high salinity in medullary interstitium.

Sorbitol causes

-defect in inositol signaling.

-depletion of myo inositol.

-decreased Na^+K^+ ATPase activity.

-decreased intracellular NADPH leading on to oxidative injury.

MECHANISM OF TUBULOINTERSTITIAL IMPAIRMENT

The impairment of the tubulointerstitium (tubulointerstitial fibrosis and tubular atrophy) is, as important as glomerulosclerosis as tubules and interstitium constitute more than 90% of kidney volume.

Tubulointerstitial fibrosis has the following characteristic features:

- inflammatory cell infiltrate which results from both activation of resident inflammatory cells and recruitment of circulating inflammatory cells.
- an increase in interstitial fibroblasts due to increased proliferation and decreased apoptosis of resident interstitial cells
- the appearance of myo fibroblasts expressing the cytoskeletal protein α-smooth muscle actin, which arise by differentiation of resident interstitial fibroblasts and infiltrating cells and via trans-differentiation.
- accumulation of extracellular matrix (ECM) as the net result of increased synthesis of ECM components and decreased ECM degradation mostly by specific metalloproteinase that are under the control of specific inhibitors.
- tubular atrophy as a consequence of apoptosis and epithelial-mesenchymal trans-differentiation (EMT) and rarefaction of peritubular capillaries.

The development of fibrosis is associated with an increase in the expression of proinflammatory, vasoconstrictive and profibrotic factors[(5)].

Renal fibrogenesis:

The initial insult leads to inflammatory response with the release of inflammatory mediators, an increase in local vascular permeability, activation of endothelial cells, extravasation of leukocytes along the endothelium, subsequent secretion of various mediators by leukocytes and tubulointerstitial cells and activation of pro-fibrotic cells. A vicious cycle of cell stress is initiated generating pro-fibrotic and pro-inflammatory mediators, leukocyte infiltration and fibrosis.

Induction and development of the inflammatory response

Leukocytes infiltrate into interstitium following gradients of chemoattractants and chemokines. The factors involved in the formation of tubulointerstitial inflammatory infiltrates are proteinuria, immune deposits, chemokines, cytokines, calcium, phosphate, metabolic acidosis, uric acid, lipids, hypoxia and reactive oxygen species.

Inflammatory cells are composed of monocytes / macrophages and lymphocytes, particularly T lymphocytes (CD4 – positive T cells and CD3 T cells carrying chemokine receptors CCR5 and CxCR3).

The pro-fibrotic factors involved in renal fibrogenesis are angiotensin II, TGF-β1, CTGF, PDGF, FGF2, EGF, ET-1, tryptase mast cell. TGF-β is the key cytokine in renal fibrogenesis.

Epithelial–mesenchymal transition:

Phenotypic conversion of epithelial cells into mesenchymal cells is known as the epithelial–mesenchymal transition. Evidence is based upon the utilization of mesenchymal marker proteins such as vimentin or S100A4, the human analogue of fibroblast specific protein – 1. It is also shown that hypoxia-inducible factor-1 (HIF-1) is the master regulator of the adaptive response controlling the expression of many genes and also stimulates EMT, which explains why hypoxia results in fibrosis and progressive renal failure. Prolonged exposure to hypoxia leads to mitochondrial injury and apoptosis consistent with the loss of tubular cells. Changes in HIF expression correlate with the extent of tubulointerstitial injury[(5)].

Proteinuria and tubulointerstitial damage

Proteinuria can damage tubulointerstitium through multiple pathways including direct tubular toxicity, changes in tubular epithelial metabolism, induced cytokine and chemokine synthesis and increased expression of adhesion molecules (Abbate). Excess protein reabsorption in proximal tubule may exceed lysosomal processing capacity leading to lysosomal rupture and direct tubular toxicity. The mechanisms include generation of chemotactic factor for macrophages, secretion of chemokines such as monocyte chemoattractant protein -1 and RANTES, and expression of fractalkine (a chemokine promoting mononuclear cell adhesion). Proteinuria also induces the secretion of TGF-β as well as that of intercellular adhesion molecule -1 and vascular adhesion molecule -1[(6)].

MEASUREMENT OF GFR:

National kidney foundation recommends that glomerular filtration rate estimation is necessary for diagnosis, screening, classification and monitoring of chronic kidney disease. Without GFR measurements, CKD may remain silent and the patient will land up in end stage renal disease.

GLOMERULAR FILTRATION RATE:

"GFR is a measure of the rate at which water and dissolved substances (low molecular weight, ultra filterable compounds) are filtered out of the blood per unit time." It provides a measure of filtering capacity of the kidneys.

$$GFR = \frac{\text{Urine Concentration} \quad X \quad \text{Urine Volume}}{\text{Plasma Concentration}}$$

Normal GFR, adjusted for body surface area, is 100-130 mL/min/1.73m^2.

Mean GFR for Indians =95.5+/-11.6mL/min. Above 40 yrs of age, GFR decreases by about 0.4–1.2 mL/min/ every year. Accurate estimation of GFR in Indians is limited due to lack of GFR estimated equation validated for our population. Indians have lower normal range of GFR than western population.

SERUM CREATININE:

Serum creatinine is used for the measurement of GFR because:

- creatinine is one of the filtration markers, whose clearance roughly equals the GFR.

- creatinine excretion is consistent among individuals and over a period of time.
- estimation of serum creatinine is accurate and consistent among clinical laboratories.

USES OF ESTIMATEDGLOMERULARFILTRATION RATE (eGFR):

- An index of functioning renal mass.
- Keeping track of changes in GFR can delineate progression of renal dysfunction.
- Predicts the time of onset of Kidney failure along with the risk of complications.
- Helps in proper dosing of drugs corrected to GFR to avoid potential drug toxicity.

CKD-EPI (CKD-epidemiology collaboration group):

- Same accuracy as that of MDRD equation at GFR < 60 mL/min/1.73m^2.
- Greater accuracy at higher GFR i.e. GFR >60mL/min./1.73$m^{2.}$ It reduces the overestimation of CKD with MDRD equation.
- Gender, age, race & serum creatinine were included.
- Different factors for black, white or other races with different serum creatinine values in both the male and female subjects.
- Better accuracy with important applications in public health and clinical practice.

CKD-EPI (IDMS CALIBRATED)

- eGFR=141*min (Serum Cr/K,1)$^{\alpha}$ *Max (Serum Cr/K,1)$^{-1.029}$ *0.993^{age} *(1.018iffemale)*1.159if black.
- K-0.7 for female, &0.9 for Male.
- α is -0.329 for female&-0.411 for Male, min indicates minimum of serum Cr/K or 1,Max-Maximumofserum Creatinine/K or 1.

WHITE OR OTHER RACE (Female)

- If serum creatinine </=0.7
- eGFR=144*(serum Cr/0.7)$^{-0.329}$*o.993$^{Age.}$
- If serum creatinine >0.7,
- e GFR=144*(serum Cr/0.7)$^{-1.209}$*0.993$^{Age.}$

WHITE OR OTHER RACE (MALE)

- If serum Cr<=0.9,
- e GFR=141*(serum Cr/0.9)$^{-0.411}$*o.993^{Agecc}
- If serum Cr>o.9,
- eGFR=141*(serum Cr/0.9)$^{-1.209}$*0.993$^{Age.}$

Klotho

Klotho is a single pass transmembrane protein that shares sequence identity with family 1 β-glycosidases. Klotho gene is one, but three isoforms of Klotho protein exist, in the transmembrane form, a soluble form which is shed, and a truncated soluble form formed by alternative splicing of Klotho mRNA. The extra

cellular domains of Klotho KL1 and KL2 type 1 β-glycosidase can be shed from the surface of the cell by membrane anchored proteolytic enzymes and liberated into circulation[7,8].

The expression of Klotho is predominantly present in proximal and distal renal tubules, in the parathyroid glands and choroid plexus in the brain[9].

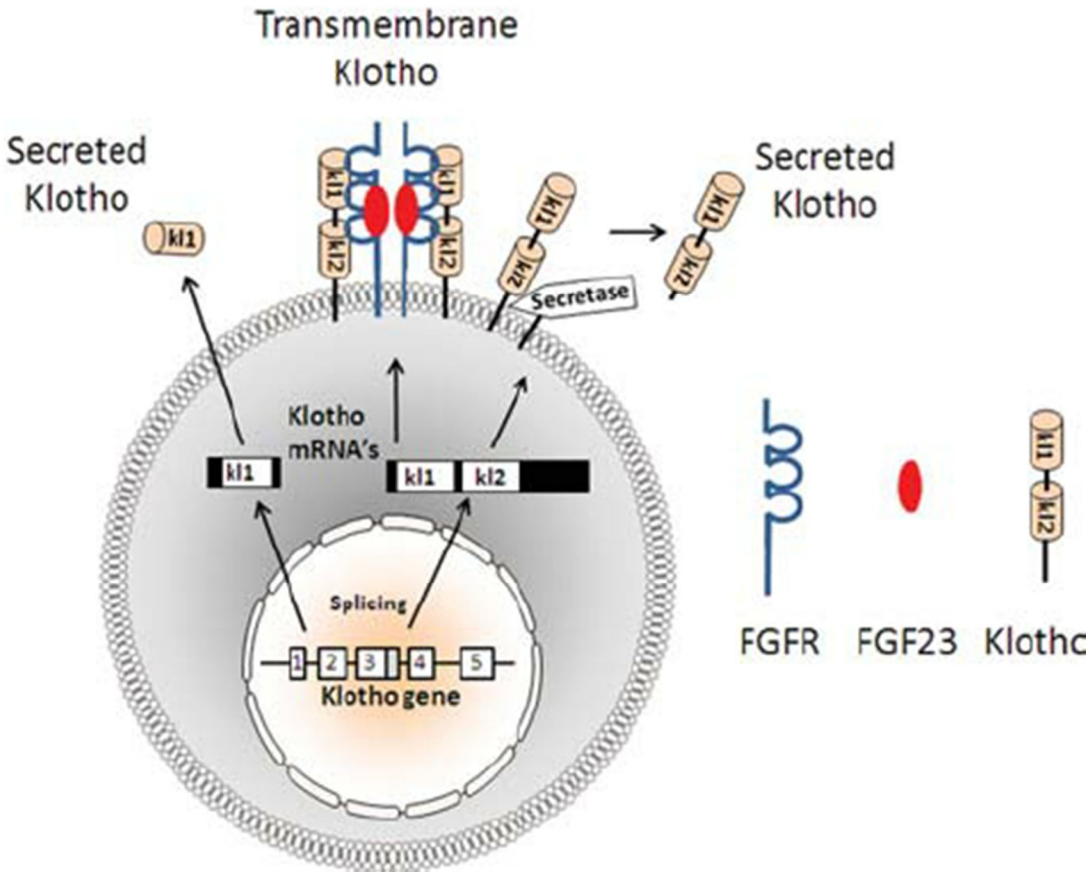


Figure 1: Schematic diagram of secreted Klotho, membrane Klotho, FGF 23 and FGF 23 receptor.

Courtesy: Orson W Moe. The emerging role of Klotho in clinical nephrology.

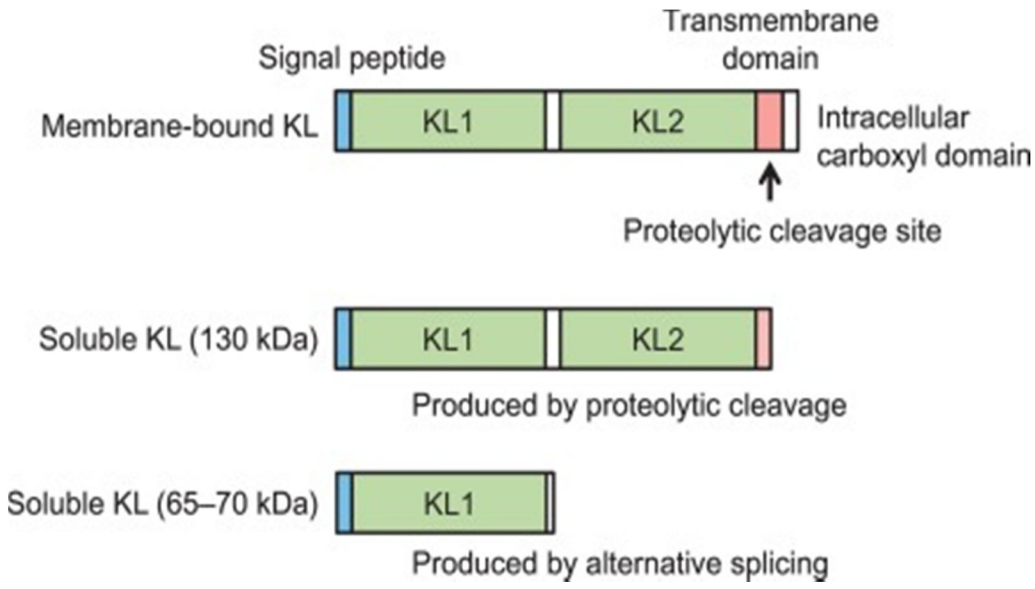


Figure 2: Production of various forms of klotho.
Courtesy : Masanobu Kawai. The FGF 23/klotho axis in the regulation of mineral and metabolic homeostasis.

The multifaceted protein Klotho is found in blood, urine, cerebrospinal fluid and plays a significant role in anti aging, energy metabolism, anti-oxidation, ion transport, regulation of PTH and vitamin D and antagonism of renin-angiotensin –aldosterone system[(10)].

Chronic kidney disease can be considered as a state of deficiency of endocrine and renal klotho. Thus it may serve as an important early biomarker and a pathogenic contributor for progression and complications in chronic kidney disease[(11)].

Klotho in kidney

Soluble α Klotho functions as an autocrine or paracrine enzyme from the intra luminal side in the kidney to modulate transporters and ion channels. α Klotho modifies the Na^+- phosphate co transporter(NaPi-2a)as a glucuronidase and the renal outer medullary K^+ and TRPV5 calcium channel as a sialidase. The terminal sialic acid residues from the N-linked glycans on the cell surface are detached by sialidase activity[(12)].

The endocytosis of transient receptor potential cation channel, subfamily 5, member 5(TRPV 5) and renal outer medullary potassium channel 1(ROMK1) is averted by secreted Klotho by modification of the N- linked glycoproteins.

Moreover, α Klotho acts from the basolateral side of membrane as a glucuronidase modifying organic cation transport[(13)].

The activity of insulin, insulin- like growth factor -1 and transforming growth factor-β1 is suppressed by secreted klotho by interacting with them or their receptors[(14)].

These enzymatic reactions are FGF 23 -independent and not attributable to α klotho's function as a co receptor.

The expression of α Klotho is maximal in the kidney, but it is not clear whether α Klotho circulating in serum is derived from the kidney. The strongest evidence to date, comes from renal tubule-specific partial deletion of α Klotho,

which causes reduced serum α Klotho levels and systemic features closely resembling the phenotype in global α Klotho deletion mice, indicating that the kidney may be the principal source of endocrine α Klotho[15,16].

The route of elimination of circulating α Klotho is less understood than its production. Animal experiments indicate the presence of intravenously injected exogenous extracellular domain of recombinant α Klotho protein in urine[17]. Transport of circulating α Klotho into the urine is through transepithelial transcytosis[16], thus making circulating α Klotho to act as luminal enzyme to modulate target proteins[18].

Thus both production and clearance of α Klotho is altered in a complex manner in chronic kidney disease.

Type 1 transmembrane proteins can be cleaved and released by "A Disintegrin And Metalloproteinase" (ADAM), a metalloproteinase, which cleaves type I transmembrane proteins to cast off membrane anchored growth factors, cytokines and receptors. In the regulation of cell signaling in physiological and pathological conditions, ADAM plays a vital role[19].

Tissue culture studies revealed that α secretase (ADAM 10/17) and β secretase inflect shedding of ectodomain to form soluble α Klotho protein by acting at two cleavage sites: adjacent to the juxtamembrane region and between the KL1 and KL2 domains[20].

α Klotho traverses across renal tubules from the basolateral to luminal side, indicated by its absence in Bowman's space and by its presence in proximal lumen[16,21].

In addition, Klotho protects the kidney from the oxidative damage[22].

Klotho in CKD

Animal studies reveal that there are striking similarities between Klotho deficient mice and mice with CKD. The features include hyperphosphatemia, elevated FGF23 levels, ectopic soft tissue calcification and diminished Klotho levels in the blood and kidney, suggesting that CKD might be a condition of Klotho deficiency[11].

In CKD patients predisposing with obstructive nephropathy, rejected transplanted kidney, diabetic nephropathy, chronic glomerulonephritis and other causes, renal Klotho RNA is remarkably diminished[23].

While clinical data in human plasma Klotho is yet to be acquired, urinary Klotho levels of CKD patients were shown to decrease at very early stage and sustainably reduced with progression of CKD[18]. Klotho levels in plasma, urine and kidney are decreased in parallel in rodent CKD models.

Overexpression of Klotho improves renal function and ameliorates renal histology, which are associated with less superoxide anion generation and lipid peroxidation, and with decreased levels of cell senescence markers, mitochondrial

DNA fragmentation and apoptosis. Thus Klotho may serve as a reno protective factor by diminishing oxidative stress, cell senescence and apoptosis[24].

The pathophysiological mechanisms underlying Klotho downregulation in CKD are more complex than just renal tissue loss. It is likely that uremic toxins and abnormal mineral metabolism contribute to downregulation of renal Klotho and subsequent decrease in blood Klotho.

Aberrant mineral metabolism

Hyperphosphatemia is a pathogenic intermediate contributing to chronic progression and complications in CKD. Phosphate overload inhibits Klotho expression in the kidney[25]. Normal mice fed with high Pi diet have strikingly decreased Klotho protein and Klotho mRNA in the kidney, while Klotho hypomorphic mice fed with low Pi diet regain the expression of Klotho partially[26]

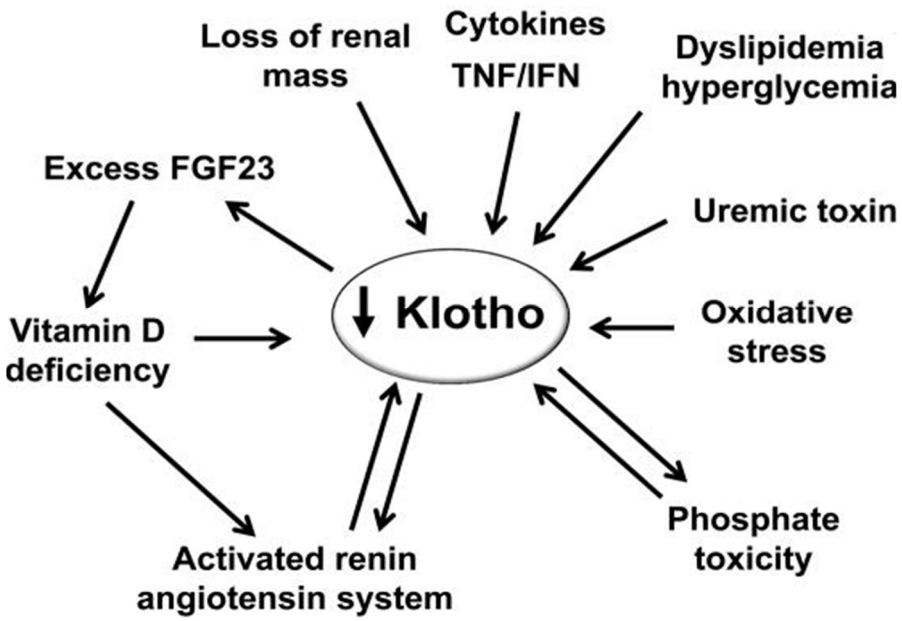


Figure 3: Mechanism of downregulation of Klotho expression in CKD

Courtesy: Hu et al. Contrib Nephrol.2013; 180:47-63.

Low 1,25- dihydroxy vitaminD3 may be an effect of loss of renal tissue and high blood FGF23 in CKD[27].Vitamin D responsive elements are found in human and rodent Klotho gene and administration of 1,25- dihydroxy vitaminD3 promotes renal Klotho expression in vivo. In vitro studies point out that 1,25-dihydroxy vitaminD3 induces membrane and secreted forms of Klotho mRNA in kidney cell lines[28].

Vitamin D receptor null mice have reduced renal Klotho mRNA expression, indicating that an interplaying network of vitamin D, phosphate and FGF 23 leading to Klotho production exists[29].

Uremic toxin

Indoxyl sulfate, a uremic toxin which accumulates in the blood in CKD, likely promotes progression and cardiovascular disease in CKD and suppresses renal Klotho mRNA in Dahl salt – resistant normotensive and hypertensive rats and induces tubulointerstitial fibrosis and increases cell senescence in renal tubular cells of Dhal salt- resistant hypertensive rats. Indoxyl sulfate may downregulate renal Klotho in CKD through stimulation of oxidative pathways and NF-κB[30].

Renin- angiotensin- aldosterone(RAA) system

Angiotensin II and aldosterone suppress Klotho expression in the kidney and kidney cell lines[31].

It was put forward that angiotensin II – induced oxidative stress leads to downregulation of Klotho expression, because free radical scavenging averts depletion of Klotho by both oxidative stress and angiotensin II infusion. The intercommunication between RAA activation, phosphate and the vitamin D – FGF 23 axis intensifies downregulation of Klotho expression in CKD[31].

Klotho's rolein progression of chronic kidney disease

Deficiency of Klotho is not only a trivial biomarker for CKD but contributing factor for CKD development, progression and complications[11].

Render renal tissues more vulnerable to injury

Cell ageing induced by Klotho deficiency is attributed by up-regulation of Wnt signal activity. The intracellular Klotho abolishes cell senescence by inhibiting retinoic acid –inducible gene 1 activated expression of IL-6 and IL-8 in vitro and in vivo[24].

Klotho deficiency in CKD would augment cell senescence caused by oxidative stress and accelerated senescence eventually leads to apoptosis. Reduction in stem cells might decline the kidney's ability to check against renal insults and impair regeneration[32].

Promote renal fibrogenesis

Renal fibrosis is a prominent histological feature in CKD and presumably plays a part in chronic progression; and TGF – β is a key player in renal fibrosis[33]. Klotho attenuates renal fibrosis by inhibiting the action of TGF- β1 on

type IITGF- β receptor. TGF-β1 – induced Smad II phosphorylation is suppressed by klotho in rat tubular epithelial cell line, putting forward that Klotho protein inhibits fibrosis mainly by inhibiting TGF- β1 signaling[34].

Contributions of Klotho deficiency to complications of chronic kidney disease

Deficient Klotho not only aggravates renal disease but also exacerbates extra renal complications in CKD

Ectopic calcification:

Extensive ectopic calcification in soft tissue observed in Klotho deficient mice is also noticed in CKD subjects, suggesting a possible pathogenic interrelation between Klotho deficiency and calcification. Amplification of Klotho by supplementation or genetic manipulation markedly inhibits vascular calcification in CKD animals[35].

Elevated plasma Pi correlates with vascular calcification in CKD and multiple pathogenic roles of high Pi have been proposed to induce or exacerbate vascular calcification. High ambient Pi induces vascular smooth muscle cells reprogramming to a phenotype resembling osteoblast and osteochondrocytein a type 3 Na^+ coupled transporters (Pit1 and Pit2) dependent manner[36].

High Pi causes cell apoptosis and consequent vesicle release for deposition on matrix. Klotho suppress this reprogramming caused by high Pi in rat cell lines in vitro[37]. Klotho also plays as an anti- inflammatory mediator in vascular endothelial cells and smooth muscle cells.

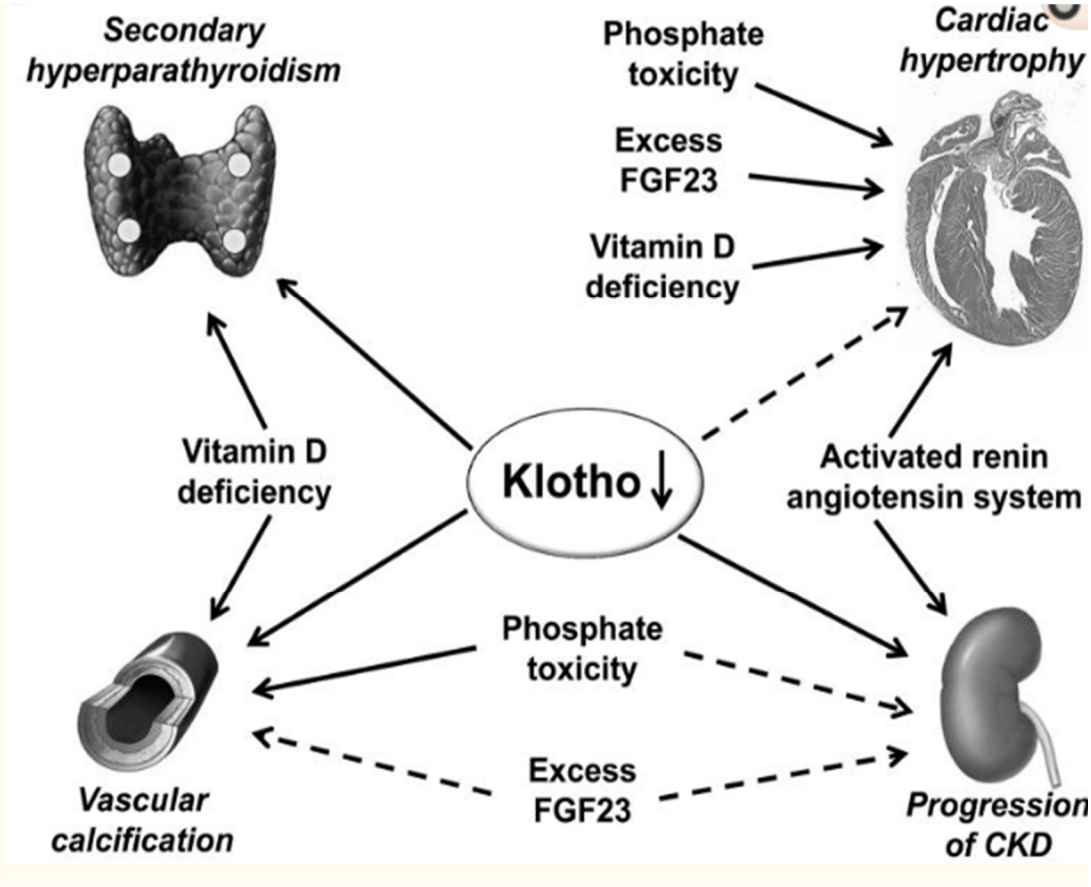


Figure 4: Pathogenic role of Klotho deficiency.

Courtesy: Hu et al. Contrib Nephrol.2013; 180:47-63.

Osteogenic/chondrogenic trans – differentiation of vascular smooth muscle cells under the influence of uremic environment including high Pi fails to respond to FGF23[(32)]. Extremely high FGF23 exerting pathological activity via stimulation of off-target genes to bring about vascular calcification in the background of low blood Klotho is one more probability[(18)].

Uremic cardiomyopathy

Cardiac hypertrophy, particularly left ventricular hypertrophy, is often encountered in CKD patients. Apart from common risk factors, novel factors including activated RAA system, vitamin D deficiency, high FGF 23concentrationand low Klotho concentration are involved in causing cardiomyopathy of CKD[38].

Klotho is expressed in the heart exclusively at the sinoatrial node.

Secondary hyperparathyroidism

Apart from abnormal mineral metabolism (Pi, Ca and vitamin D), high FGF23 and low Klotho contribute to secondary hyperparathyroidism. The plasma level and activity of FGF23 is raised and FGFR1 and Klotho expression is reduced in parathyroid glands of CKD patients[39].

Under physiological milieu with normal Klotho and FGFR expression, FGF23 reduces PTH production, raises parathyroid calcium –sensing receptor and the vitamin D receptor expression and suppression of cell proliferation. In the uremic milieu with decreased Klotho and FGFR1 expression in parathyroid glands, FGF23 no longer exerts its inhibitory effect on the gland and fails to raise calcium sensing receptor and vitamin D receptor[40].

Fibroblast growth factor 23

FGF 23 is a protein encoded by the FGF23 gene on chromosome 12p13.3 incorporating 251 aminoacids[41]. “After cleavage of the native protein the signal

sequence containing 24 aminoacids and O-glycosylation by UDP-N-acetyl-alpha-D-galactosamine: polypeptide N-acetyl galactosaminyl transferase 3 (GALNT3), the mature protein, FGF23 (25-251), is secreted into the circulation"[42,43].

FGF23 is deficient in heparan-sulfate (HS) binding -motifs, which reduces the affinity of matrix binding and facilitates the ligand to function in an endocrine way[44].

Four specific genes encode FGF receptors (FGFR1-FGFR4). All the four receptors share an almost identical domain structure. The three immunoglobulin – like domains (D1-D3) form the extracellular domain, followed by a single pass transmembrane domain and an intracellular domain possessing tyrosine kinase activity[45].

Studies reveal that in all the three FGF 19 subfamily members, an important residue for FGFR binding is replaced, so these FGFs have intrinsically reduced affinity for their cognate receptors. Decreased receptor binding affinity along with the low HS binding affinity of these FGFs is the mechanism behind the dependence of these ligands on Klotho proteins to act in their target tissues[45].

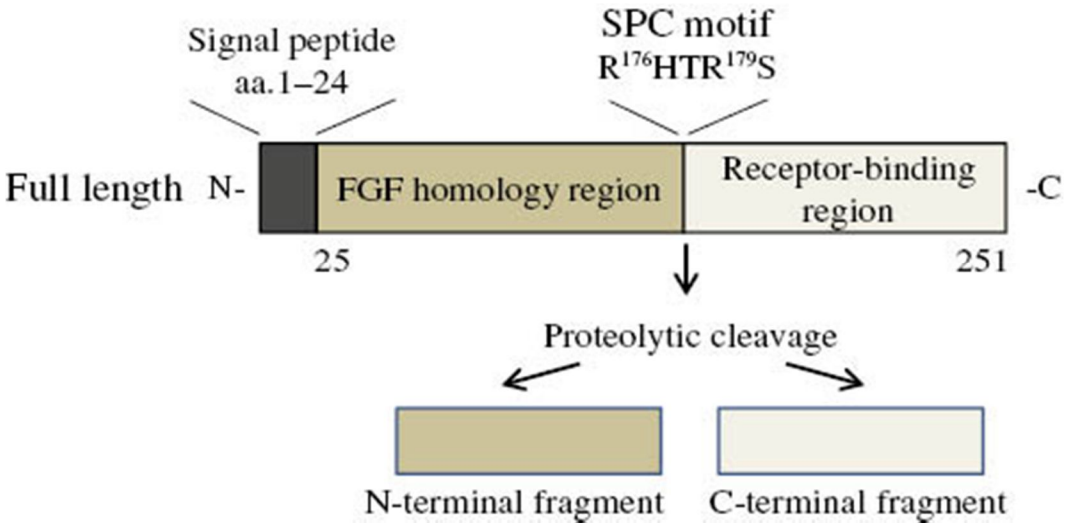


Figure 5: Structure of FGF 23 and its cleavage site.

Courtesy : Masanobu Kawai. The FGF 23/Klotho axis in the regulation of mineral and metabolic homeostasis.

FGF23 depends on Klotho as a coreceptor. "Immuno-precipitation experiments, surface plasmon resonance (SPR) spectroscopy and functional assays measuring the mitogenic response of BaF3 cells or activation of the MAPK-pathway in HEK293 cells have shown that Klotho forms a ternary complex with FGF23and either FGFR1c, FGFR2c, FGFR3c or FGFR4"[(46)].

Studies in literature using neutralizing ant-iFGF23 antibodies point out that the N-terminal portion ofFGF23 interacts with FGFR1c, while the C- terminus binds to Klotho and both these aspects of intercommunication are crucial for bioactivity in vitro and in vivo[(47)].

A study using mice with kidney specific deletion of FGFR1 demonstrates that FGFR1 modulates the phosphaturic effects of FGF23[48].

FGF23 utilizes a non-canonical signal transduction pathway at the proximal tubules or it promotes the secretion of an intermediary phosphatonin in the distal tubule, which has its effects in a paracrine mode on the proximal tubules[49].

Phosphate and vitamin D metabolism is influenced byFGF23, a member of the fibroblast growth factor family. FGF 23 is a bone derived hormone produced by osteoblasts and osteocytes in response to vitamin D and raised phosphate. FGF 23 is basically a phosphaturic hormone, controlling apical membrane type 2 a sodium – phosphate cotransporters in proximal tubules by acting through FGF receptors and the coreceptor Klotho.

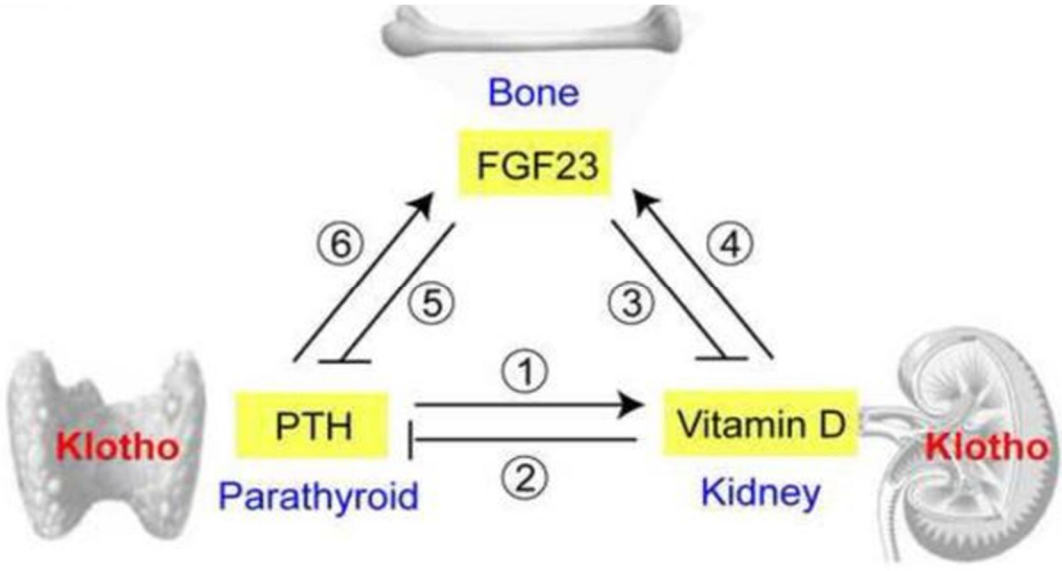


Figure 6: Endocrine regulation of phosphate homeostasis.

Courtesy : John et al. Am J Kidney Dis.2011 July; 58(1):127-134

1. PTH stimulates vitamin D synthesis.
2. Vitamin D by negative feedback decreases PTH.
3. FGF23 reduces vitamin D synthesis.
4. Vitamin D increases FGF 23 expression in bone by negative feedback.
5. FGF 23 reduces PTH synthesis.
6. PTH increases FGF 23 expression by negative feedback.

1 α-hydroxylase expression is suppressed by FGF 23 and thus the synthesis of the active vitamin D hormone is decreased. It also stimulates the catabolic 24-hydroxylase and inhibits parathyroid hormone secretion[50].

Healthy individuals are maintaining their serum phosphate in a narrow range because FGF23 levels adjust in parallel with the amount of dietary phosphate intake. FGF23 levels increase in feedback to high phosphate intake, promoting greater urinary fractional excretion of phosphate and by lowering 1,25-dihydroxyvitamin D levels, thus reducing the efficiency of intestinal phosphate absorption[51].

A classic negative feedback loop exists between vitamin D and FGF 23, thus 1,25-dihydroxy vitamin D stimulates FGF 23 secretion and FGF 23 suppresses the levels of 1,25-dihydroxy vitamin D[52]. FGF 23 transcription is regulated by vitamin D response element in the FGF 23 promoter site in such a way that some amount of vitamin D activity is essential for FGF 23production[53].

The serum phosphate levels are determined by the balance between absorption of phosphate in the intestine from the diet, storage in the skeleton and excretion through urine.

Phosphate reabsorption from the urine in the proximal renal tubules via type 2 and type 3 sodium phosphate co-transporters plays a significant role. Renal phosphate reabsorption is under tight hormonal regulation by parathyroid hormone and fibroblast growth factor 23.

FGF 23and mortality

Elevated FGF 23 levels were independently correlated with higher risk of mortality[54]. Kidney transplant recipients with raised FGF 23 levels were associated with greater risk of mortality, allograft loss and their composite[55].

FGF 23 and progression of CKD

Elevated FGF 23 is a potentially significant risk factor for ESRD[56].

FGF 23 and cardiovascular disease events

Elevated FGF 23was associated with greater risk of myocardial infarction, stroke, coronary, carotid or lower limb revascularization, amputation and death[57].

Therapeutic strategies to lower FGF 23 levels

Reducing dietary phosphate intake lowers FGF 23 levels in healthy individuals[58]. In a randomized control trial, assignment of a 750-mg phosphate diet did not significantly lower FGF 23 levels but the 1500 mg diet raised FGF 23

levels in a considerable number of participants, putting forward that dietary phosphate ingestion contributes to dynamic changes in FGF 23 levels in CKD[59].

Administration of phosphate binders such as lanthanum, sevelamer and aluminium – magnesium lowered FGF 23 levels in healthy subjects, hyperphosphatemic ESRD patients and CKD patients with phosphate levels normal or elevated[60,61].

FGF 23 in bone:

Fibroblast growth factor 23 has physiologically significant functions in bone on bone mineralization and on hematopoiesis[62]. FGF 23 is a strong suppressor of tissue non-specific alkaline phosphatase (TNAP) mRNA transcriptionin bone in a Klotho independent manner. TNAP is a key factor for the regulation of bone mineralization by cleaving the mineralization inhibitor pyrophosphate synthesized by osteoblasts to prevent premature mineralization of osteoid[63].

Parathyroid cell resistance to fibroblast growth factor 23 in secondary hyperparathyroidism of chronic kidney disease:

FGF 23 exerts its action on the parathyroid through its receptor, Klotho-FGFR1c, to activate the MAPK pathway and lower PTH expression. High levels of both FGF 23 and PTH are observed in CKD. In CKD there is decreased expression of parathyroid gland Klotho and FGFR1 mRNA and these changes contribute to the resistance of the parathyroid to high levels of FGF 23[64,65].

Calcium and phosphate homeostasis in chronic kidney disease

Calcium and phosphate are crucial for normal cardiovascular and neuromuscular function and for many enzyme mediated and cellular signaling activity. Calcium and phosphate provide structural framework to the skeleton, which is the primary storehouse of mineral ions in the body[66].

When the concentration of ionized calcium decreases, parathyroid hormone stimulates release of calcium from skeletal reserves and stimulates conversion of 25-hydroxy vitamin D to biologically active1,25- dihydroxy vitamin D, which enhances gastrointestinal calcium absorption[66].

Resorption of bone mediated by PTH releases phosphate into the extracellular fluid. Excessive phosphate in circulation is excreted by the kidney in urine in response to PTH and fibroblast growth factor 23 acting on FGF receptor-Klotho co-receptor complexes[67].

“As part of an intricate set of feedback loops that tightly regulate calcium and phosphate homeostasis, both PTH and1,25-dihydroxy vitamin D stimulate FGF23 secretionandFGF23 is the primary negative regulator of 1,25- dihydroxy vitamin D production”[66].

There is an interruption of calcium and phosphate homeostasis early in the course of chronic kidney disease.

Human and animal data suggest increased FGF23 level is the earliest detectable alteration[68]. Rising FGF23 levels suppress renal production of 1,25-dihydroxy vitamin D, which reduces Klotho expression.

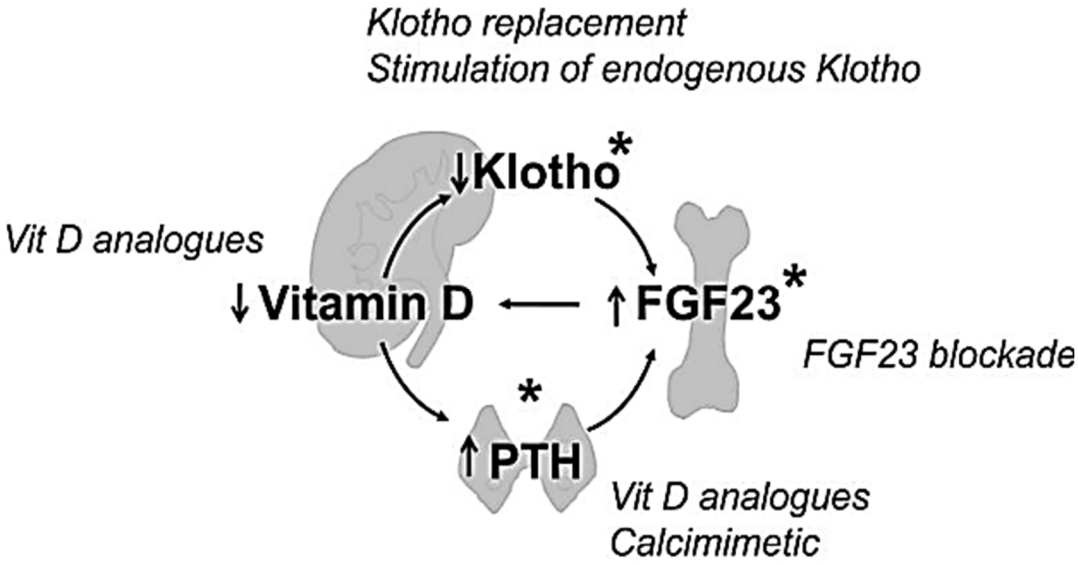


Figure 7: Proposed therapeutic strategies in CKD

Courtesy: Hu et al. Contrib Nephrol.2013; 180:47-63.

Responding to the decrease in ionized calcium concentration due to inefficient gastrointestinal calcium absorption, the parathyroid glands stimulate PTH secretion, which is augmented by the release of the glands from feedback control by reduced 1,25-dihydroxy vitamin D levels[69]. Secondary hyperparathyroidism resulting in constitutive bone resorption plays a major role in bone disease in CKD[70].

PTH rises in CKD as a secondary response to calcium depletion and FGF 23 rises as a secondary response to phosphate retention[71].

Healthy kidney excretes approximately two thirds of the dietary phosphate load and even with declining kidney function in CKD, this excretion is comparatively well maintained.

The serum phosphate concentrations were found to be in the normal range until a GFR of 30 mL/min; on further decrease of GFR the phosphate levels begin to rise beyond the upper normal range and are found to become inversely related to GFR, while it is still unclear when serum phosphate levels tend to rise "within" the normal range in CKD[(72)].

Serum phosphate levels above 3.5 mg/dL have already been proved to be significantly associated with mortality. Mortality risk progressively increased with each 0.5-mg/dL rise in phosphate serum levels[(73)].

There is delayed onset of hyperphosphatemia in CKD probably due to counter regulatory actions of FGF 23 and PTH. Both the hormones cause phosphaturia when elevated and apparently stimulated as early as in CKD stage 3, corresponding to vitamin D status and dietary phosphate intake.

Rise in serum FGF 23 levels was analogues to rise in renal fractional phosphate excretion, counterchecking increase in phosphate retention and subsequent hyperphosphatemia[(74)].

The positive phosphate balance brings about an osteocyte "phosphate sensor" communicating the release of FGF 23. PTH and FGF 23 production as well as release are directly interconnected. FGF 23 suppresses PTH release via

stimulating the dimeric Klotho/FGF 23 – receptor at the parathyroid gland. PTH may vice versa inhibit FGF 23 secretion from the osteocyte[72].

Under normal physiological condition, metabolism of calcium is regulated through hormones in a three – tissue axis of intestine, kidney and bone to maintain serum ionized calcium within a narrow range.

The maintenance of serum phosphate within normal limits in early stage CKD is not the physiological fact because it is maintained within normal limits at the expense of PTH increase[75].Variation in serum levels is a late stage consequence of a reduction in urinary total excretion as renal function declines, when the mechanism is no longer compensated by the increase of FEP induced by PTH[76].

Serum calcium levels are involved in hyperparathyroidism progression but play an important role in the late stages when calcium begins to decrease[76].

The two chief hormones involved are 1,25- vitaminD /calcitriol and PTH. Low serum ionized calcium is sensed by calcium sensing receptors on the parathyroid gland which in turn stimulates PTH synthesis and secretion.

Aim of the study

AIM OF THE STUDY

- To estimate the level of serum soluble α Klotho and FGF 23 levels in CKD patients.
- To establish its role in chronic kidney disease - mineral bone disease.

Materials and Methods

MATERIALS AND METHODS

STUDY CENTRE

The study was carried out during the period April 2017 – August 2017 at Madras Medical College and Rajiv Gandhi government general hospital at the following departments

- Institute of Biochemistry
- Institute of Nephrology

This is a Case Control study and was conducted after getting clearance from the institutional ethics committee.

GROUP A:

60 unrelated individuals diagnosed with CKD (KDOQI guidelines) attending the outpatient department of nephrology were included in the study after getting informed consent.

Inclusion criteria

Eligible CKD patients who gave informed consent, Age 15-60 yrs on conservative therapy

Exclusion criteria

Acute kidney disease, patients on renal replacement therapy

GROUP B:

30 age-and-sex-matched healthy subjects who were employees in the institution and attendants accompanying the patients were included in the study after obtaining informed consent.

Inclusion criteria

Healthy subjects with normal renal function and no evidence of acute or chronic underlying illness.

Methodology:

- Serum Urea-spectrophotometric method
- Serum Creatinine-spectrophotometric method
- Serum Calcium-spectrophotometric method
- Serum Phosphate-spectrophotometric method
- Serum FGF 23-ELISA
- Serum Soluble α klotho-ELISA
- Serum PTH-ECLIA
- Spot urine protein creatinine ratio-spectrophotometric method

Sample size

- Group A- CKD patients -60,
- Group B- Healthy subjects -30

Serum soluble α Klotho estimation by ELISA

Test principle

The kit employs a double antibody sandwich enzyme linked immunosorbent assay (ELISA) to assess the level of human Klotho in samples. Add Klotho to monoclonal antibody enzyme well which is pre-coated with human Klotho monoclonal antibody; incubate ; then, add Klotho antibodies labeled with biotin and combined with streptavidin – HRP to form immune complex; then carry out incubation and washing again to remove the uncombined enzyme. Then add chromogen solution A, B. The color of the liquid changes into blue and at the effect of acid, the color finally becomes yellow. The color and the concentration of the human substance Klotho were positively correlated.

Sample collection and storage:

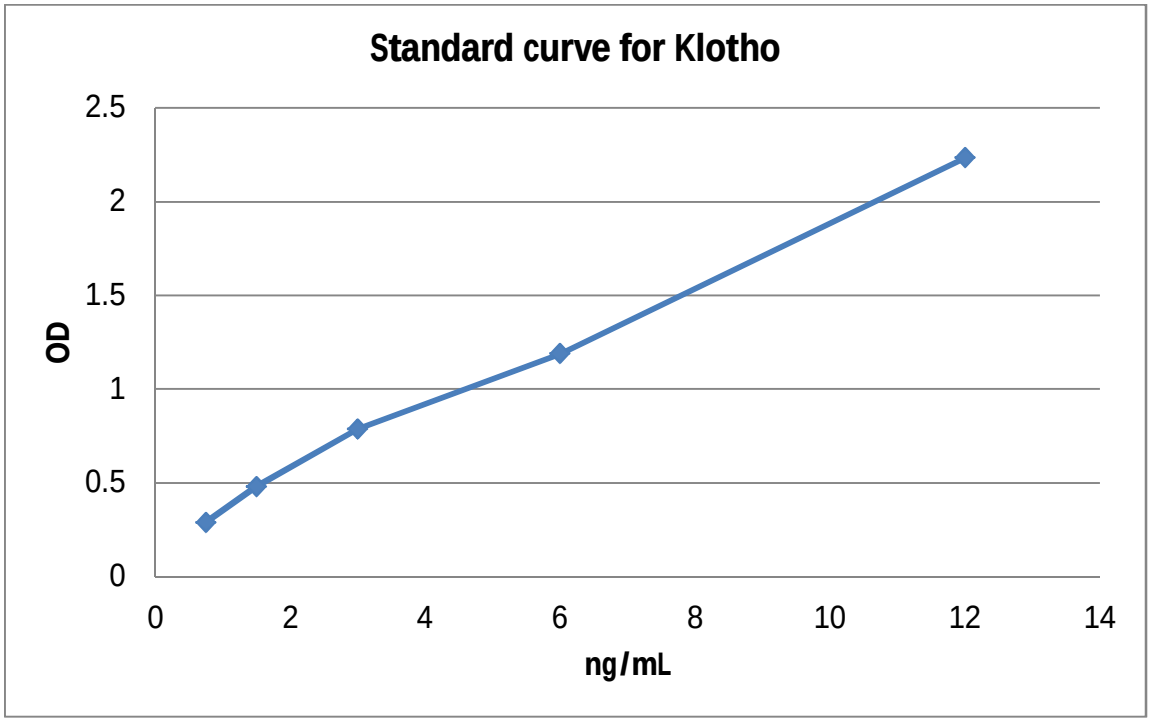
Blood samples were collected, allowed to clot for 20 minutes at room temperature. The tube is then centrifuged for 20 minutes at 3000 rpm. Tubes are then taken out and serum separated and aliquoted into 4 aliquots. Sample is stored at – 20̊ C.

Assay procedure

Kit is taken out from the 2-8̊ environment and kept at ambient temperature for 30 minutes.

Standard dilution

12 ng/mL	Standard No. 5	120μL original standard + 120μL standard diluents
6 ng/mL	Standard No. 4	120μL Standard No.5 + 120μL standard diluents
3 ng/mL	Standard No. 3	120μL Standard No.4 + 120μL standard diluents
1.5 ng/mL	Standard No. 2	120μL Standard No.3 + 120μL standard diluents
0.75 ng/mL	Standard No. 1	120μL Standard No.2 + 120μL standard diluents



Injection of samples:

- Blank well: Do not add samples and Klotho antibody labeled with biotin, streptavidin- HRP; only chromogen solution A, B and stop solution are added.
- Standard wells: add standard 50μL, streptavidin – HRP 50μL (since the standard already has combined biotin antibody, it is not necessary to add the antibody)
- Test well: Add sample 40μL and then add both Klotho antibody 10μL and streptavidin – HRP 50μL.
- Seal the plate with sealing membrane for gentle shaking. The plate is then incubated at 37° C for I hour.
- Wash solution preparation: dilute 30 times the wash concentrate with distilled water.

Wash procedure:

- The plate is removed from the incubator, membrane is carefully removed and the liquid is carefully drained from the plate.
- The plate is washed using an automated washer.
- Then add chromogen solution A 50μL, then chromogen solution B 50μL to each well.
- Incubate for 10 minutes away from light.
- Add stop solution 50μL into each well to arrest the reaction (the blue changes into yellow immediately)

Final measurement:

Take blank well as zero, measure the optical density under 450 nm wavelength, which is carried out within 15 minutes after adding the stop solution.

Serum Fibroblast growth factor 23 estimation by ELISA

Test principle:

The kit employs a double antibody sandwich enzyme linked immunosorbentassay (ELISA) to assay the level of Human fibroblast growth factor- 23 in samples. Add fibroblast growth factor 23 to monoclonal antibody enzyme well which is pre coated with human fibroblast growth factor 23 monoclonal antibody; incubate; then add fibroblast growth factor 23 antibodies labeled with biotin and combined with streptavidin – HRP to form immune complex; then carry out incubation and washing again to remove the uncombined enzyme. Then add chromogen solution A,B, the color of the liquid changes into blue, at the effect of acid, the colour finally becomes yellow. The chroma of color and the concentration of the human substance fibroblast growth factor – 23 of sample were positively correlated.

Sample collection and storage:

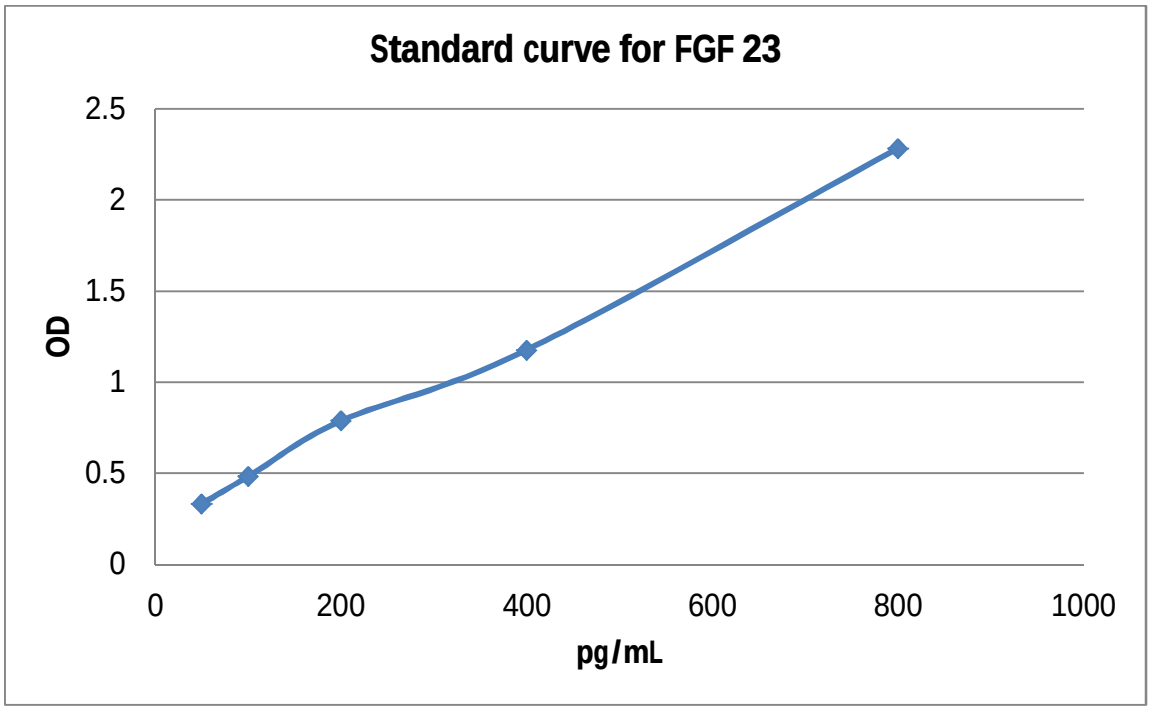
Samples are collected and are allowed to clot for 20 minutes at room temperature. The tube is then centrifuged for 20 minutes at 3000 rpm. Tubes are then taken out and serum separated and aliquot into 4 aliquots. Sample is stored at – 20° C.

Assay procedure

Kit is taken out from the 2-8° C storage and kept at ambient temperature for 30 minutes.

Standard dilution:

800pg/mL	Standard No.5	120µL original standard + 120µL standard diluents
400pg/mL	Standard No.4	120µL standard No.5 + 120µL standard diluents
200pg/mL	Standard No.3	120µL standard No.4 + 120µL standard diluent
100pg/mL	Standard No.2	120µL standard No.3 + 120µL standard diluents
50pg/mL	Standard No.1	120µL standard No.2 + 120µL standard diluents



Injection of samples:

- Blank well: Do not add samples and FGF-23 antibody labeled with biotin, streptavidin- HRP, only chromogen solution A, B and stop solution are added.

- Standard wells: Add standard 50μL, streptavidin – HRP 50μL (since the standard already has combined biotin antibody, it is not necessary to add the antibody)

- Test wells: Add sample 40μL and then add both FGF-23 antibody 10μL and streptavidin – HRP 50μL.

- Seal the plate with sealing membrane for gentle shaking. The plate is then incubated at 37C for I hour.

- Wash solution preparation: dilute 30 times the wash concentrate with distilled water.

Wash procedure:

- The plate is removed from the incubator, membrane is carefully removed and the liquid is carefully drained from the plate.
- The plate is washed using an automated washer.
- Then add chromogen solution A 50μL, then chromogen solution B 50μL to each well.
- Incubate for 10 minutes away from light.

- Add stop solution 50µL into each well to arrest the reaction (the blue changes into yellow immediately)

Final measurement:

- Take blank well as zero, measure the optical density under 450 nm is carried out within 15 minutes after adding the stop solution.

Creatinine estimation:

Method: Jaffe‘s kinetic method (using IDMS traceable calibrator)

Principle: In alkaline environment, creatinine forms a yellow –orange complex with picrate. The rate of formation of coloured complex is proportional to the creatinine concentration in the specimen. The assay uses rate blanking to minimize interference by bilirubin. To correct for non-specific reaction caused by serum/plasma pseudo-creatinine chromogens, including proteins and ketones, the results are corrected by (-0.3mg/dL).

$$\text{Creatinine + picric acid} \xrightarrow{\text{Alkaline pH}} \text{yellow orange complex}$$

Reagents:

R1 – potassium hydroxide900mmol/L; phosphate 135 mmol/L

R2- picric acid 38mmol/L; non reactive buffer

Reference range: 0.5 to 1.3 mg/dL

Urea estimation:

Method: Kinetic method with urease and glutamate dehydrogenase.

Principle: Urea is hydrolyzed by urease to form ammonium and carbonate.

$$\text{Urea} + 2H_2O \xrightarrow{\text{urease}} 2NH_4^+ + CO_3^{2}$$

In the second reaction 2-oxoglutarate reacts with ammonium in the presence of glutamate dehydrogenase (GLDH) and the coenzyme NADH to produce L-glutamate. In this reaction two moles of NADH are oxidized to NAD+ for each mole of urea hydrolyzed.

$$NH_4^+ + \text{2-oxoglutarate} + \text{NADH} \xrightarrow{\text{Glutamate dehydrogenase}} \text{L- glutamate} + NAD^+ + H_2O$$

The rate of decrease in the NADH concentration is directly proportional to the urea concentration in the specimen and is measured photometrically.

Reagents:

R1 – NaCl 9%

R2 – TRIS buffer 220 mmol/L; 2-oxoglutarate 73mmol/L; NADH 2.5 mmol/L; ADP 6.5 mmol/L; urease –jackbean; GLDH >80µkat/L

Reference range: 15 to 40 mg/dL

Calcium estimation:

Method: Arsenazo III

Principle: Arsenazo III combines with calcium ions at pH 6.5 to form a colored chromophore, the absorbance of which is measured at 650 nm and is proportional to calcium concentration.

Reagents :

Arsenazo III 0.20 mmol/L; buffer pH 6.75-100mmol/L

Reference range: 8.6 to 10.2 mg/dL

Phosphate estimation:

Method: Molybdate UV method

Principle: Inorganic phosphate combines with ammonium molybdate in the presence of strong acid (H_2SO_4) to form phosphomolybdate. The formation of reduced phosphomolybdate is measured at 340 nm and is directly proportional to the inorganic phosphate present in the sample.

Phosphate + ammonium $\longrightarrow$ ammonium phosphomolybdate

Reagents:

R1 – sulfuric acid 0.36 mol/L

R2- ammonium molybdate 3.5 mmol/L; sodium chloride 150 mmol/L

Reference range: 2.5 to 4.5 mg/dL

Parathyroid hormone estimation:

The biologically active N –terminal fragment has a half of only few minutes. Selective measurement of the intact parathyroid hormone allows direct ascertainment of the secretary activity of the parathyroid glands.

Method: ECLIA

Principle: The assay employs a sandwich test principle in which a biotinylated monoclonal antibody reacts with the N – terminal fragment (1-37) and a monoclonal antibody labeled with a ruthenium complex reacts with the c-terminal fragment.

The antibodies used in the assay are reactive with epitopes in the amino acid regions 26-32 and 37-42.

Reagents:

M – streptavidin coated microparticles 0.72mg/mL

R1-biotinylated monoclonal anti- PTH antibody (mouse)

R2-monoclonal anti PTH antibody (mouse) labeled with ruthenium complex.

Reference range: 10 to 65 pg/mL

Urine protein creatinine ratio estimation:

- Spot protein creatinine ratio was performed in the urine of subjects.
- Urine protein estimated by using pyrogalol red method.
- Urine creatinine estimated by Jaffe's method.

Statistical Analysis

STATISTICAL ANALYSIS

- Data was analyzed using SPSS software 20.00 version and P value less than 0.05 was considered significant and less than 0.001 highly significant.

- Chi square test was used for analysis of gender

- Student t-test was used for analysis of age, Klotho, FGF23, PTH, creatinine, urea, calcium, phosphate and urine PCR

- Correlation of parameters between Klotho and FGF 23, eGFR and Klotho and eGFR and FGF 23 were analyzed by Pearson correlation analysis.

- ROC curves for Klotho and FGF23 were plotted using SPSS software.

- Multiple regression analysis was performed to evaluate relationship between eGFR and other parameters (Klotho, FGF23, creatinine, urea, PTH, calcium, phosphate and urine PCR)

Results

MASTER CHART - CASES CKD

S.NO	AGE	SEX	KLOTHO (ng/mL)	FGF 23 (pg/mL)	CREATININE (mg/dL)	UREA (mg/dL)	eGFR	CALCIUM (mg/dL)	PHOSPHATE (mg/dL)	PTH (pg/mL)	PCR	DIAGNOSIS
1	52	M	1.42	647	12.3	178	5	5.2	7	350	1.1	SHT
2	53	M	1.12	598	10.5	124	6	8.1	5.9	124	3.1	DM/CAD
3	55	M	1.58	526	7.2	116	9	6.2	3.6	197	0.4	DM/SHT
4	53	M	2.08	402	6.8	123	10	7.1	3.4	123	6.3	DM/SHT
5	50	M	1.9	711	6.1	106	11	7.8	3.9	177	1.6	SHT
6	53	F	2.03	552	5	64	11	7.8	6.4	236	0.58	DM
7	60	f	3.53	408	4.5	96	12	8.4	5.4	135	2	DM/SHT
8	47	M	2.29	336	5.6	62	13	8.6	3.1	73	0.82	PT
9	50	M	3.67	452	4.8	87	15	8.2	3.8	25	1.1	SHT/SNAKE BITE
10	61	F	2.14	402	3.6	69	15	7.6	4.5	134	0.55	SHT
11	62	F	4.36	270	3.5	107	15	6.9	4.6	417	6.6	DM/SHT
12	50	M	3.29	492	4.8	81	15	9	3.7	89	4.9	CIN
13	52	F	5.04	294	3.4	65	17	9.4	4.6	65	2	DM/SHT
14	40	F	3.07	950	3.7	122	17	7.6	7.1	626	6.2	DM/SHT
15	30	M	3.97	380	4.7	76	18	7.6	4	181	1.9	SHT
16	64	F	5.38	264	3.1	90	18	8.3	3.8	90	0.05	HT/CIN/MN
17	45	F	3.29	505	3.4	95	18	7.4	3.7	275	0.73	DM
18	60	M	2.49	379	4	82	18	7.3	4.7	126	13.3	DM/SHT
19	55	M	2.73	322	3.9	75	19	8.2	2.1	75	6.2	DM/SHT
20	39	M	2.32	511	3.5	29	24	8.7	3.2	42	0.8	YOUNG STROKE/HT
21	54	M	3.49	225	3.1	60	25	7.8	4.3	60	9.6	DM/SHT
22	60	F	2.36	370	2.4	53	25	7.6	4	62	5.7	SHT/CIN
23	38	F	3.71	281	2.3	51	30	8.9	4.2	51	0.54	SHT
24	65	M	2.29	520	2.5	79	30	9	2.8	103	1.3	DM/SHT
25	55	F	1.86	573	2.1	33	30	7.1	2.2	340	0.45	DM/SHT
26	45	M	3.38	241	2.6	91	33	7.3	5.9	29	0.89	DM
27	75	M	3.04	272	2.2	77	33	9.2	3.3	10	0.79	DM
28	68	M	7.49	225	2.3	52	33	7.3	2.8	52	0.6	SHT
29	49	M	3.06	261	2.5	59	34	8.5	2.7	53	0.81	DM/SHT
30	54	M	3.27	233	2.2	38	38	7.4	2	45	2.3	IGA NEP
31	58	F	0.985	278	1.7	39	38	10.4	4.7	88	4.6	DM
32	61	F	5.55	374	1.5	47	40	8.2	2.9	44	0.08	DM / HYPOTHYROIDISM

S.NO	AGE	SEX	KLOTHO (ng/mL)	FGF 23 (pg/mL)	CREATININE (mg/dL)	UREA (mg/dL)	eGFR	CALCIUM (mg/dL)	PHOSPHATE (mg/dL)	PTH (pg/mL)	PCR	DIAGNOSIS
33	47	F	3.71	298	1.7	51	41	9	3.7	35	0.03	DM/SHT
34	25	M	3.82	236	2.4	33	42	8.9	1.6	26	0.62	IGA NEP
35	35	F	3.28	277	1.8	35	42	8.5	2.6	25	2.6	SHT
36	54	F	3.78	309	1.6	38	42	9	4	55	0.66	DM/FSGS
37	74	F	1.12	588	1.4	60	43	7.9	4.2	94	0.75	SHT
38	63	M	3.18	185	1.9	25	43	7	2.5	130	0.9	DM/SHT
39	52	F	4.34	155	1.5	40	46	8.2	3	27	0.04	CIN
40	52	F	5.09	363	1.4	35	50	9.4	3.3	33	6.1	DM/SHT
41	58	M	2.26	230	1.7	46	50	7.6	3.5	13	1.3	DM
42	70	F	1.72	214	1.2	27	53	8.6	3	44	1.3	DM
43	44	M	2.16	242	1.7	69	56	8.4	3.4	38	0.11	SHT
44	57	F	3.69	222	1.1	28	65	8.9	3	41	0.59	DM/SHT
45	55	F	4.78	211	1.1	32	65	10.6	4.5	23	0.79	ANALGESIC ABUSE
46	18	M	4.11	224	1.7	40	67	10	3.9	25	0.03	ATN/CKD/POISONING
47	60	M	3.62	192	1.3	34	69	10.5	4.1	34	0.54	SHT
48	30	F	3.31	237	1.2	32	70	7.9	3.2	17	0.04	IGA NEP
49	65	M	6.2	267	1.2	24	73	8.4	3.1	24	0.13	DM
50	42	M	3.44	302	1.3	34	78	10.9	3.5	11	0.03	DM
51	42	M	5.18	280	1.3	42	78	8.2	2.3	17	5.1	DM/SHT/IGA
52	52	M	3.72	182	1.2	29	80	8.6	2.8	32	0.9	DM/SHT
53	40	F	6.85	274	1	20	82	8.1	2.6	20	9.1	DN / HYPOTHYROIDISM
54	67	F	3.97	233	0.8	25	88	8.8	3.1	32	0.82	DM/SHT
55	47	M	3.37	230	1.1	20	92	9.5	2.8	21	0.03	IGA NEP
56	42	M	3.29	146	1.1	34	95	8.7	2.7	42	8.09	SHT
57	49	F	4.72	231	0.8	27	100	8.7	3.6	15.2	0.03	DM
58	65	M	1.62	261	0.8	32	109	9.5	3.5	17	0.03	DM
59	48	M	2.8	202	0.9	18	117	8.3	2.8	12	0.03	DM/SHT
60	35	F	2.31	185	0.7	18	130	7.9	3.1	9	1.9	IGA NEP

MASTER CHART - CONTROLS

S.NO	AGE	SEX	KLOTHO (ng/mL)	FGF 23 (pg/mL)	CREATININE (mg/dL)	UREA (mg/dL)	eGFR	CALCIUM (mg/dL)	PHOSPHATE (mg/dL)	PTH (pg/mL)	PCR
1	42	F	5.9	239	0.6	28	130	10.3	3.1	20	0.02
2	77	F	3.46	297	0.8	32	82	10.1	3	28	0.08
3	72	M	11.39	189	0.8	19	103	10	3.5	55	0.08
4	58	M	4.97	217	1.3	38	70	9.3	3.2	28	0.06
5	43	M	9.53	206	1	35	106	9.4	3.7	16	0.03
6	53	M	8.03	207	0.8	28	118	10.1	3.8	23	0.05
7	57	M	4.13	268	1	42	96	9.1	3.7	41	0.08
8	53	F	8.26	202	0.8	14	98	10	4.2	35	0.19
9	42	F	6.02	260	0.7	18	124	9.5	3.6	34	0.18
10	45	F	5.28	238	0.9	12	89	9	3.6	27	0.04
11	55	M	4.34	227	1	28	98	9.5	3.6	45	0.06
12	60	M	3.8	246	0.9	32	107	9.8	3.2	44	0.07
13	55	M	11.41	142	0.7	37	123	10	3.9	20	0.06
14	43	F	6.34	204	0.6	20	129	9	4.1	30	0.13
15	62	M	4.19	166	0.6	29	125	9.3	4.1	49	0.1
16	55	M	5.31	111	0.7	26	123	9.3	3.2	17	0.05
17	43	M	6.17	258	0.7	22	134	8.9	4.1	15	0.08
18	57	M	4.24	241	0.7	41	121	8.6	3.9	15	0.07
19	55	M	4.19	243	0.8	31	117	9.7	3.7	17	0.06
20	54	F	4.44	239	1	28	74	9.9	3.7	23	0.13
21	52	M	4.35	226	0.8	35	119	9.7	2.9	7	0.19
22	57	M	4.55	255	0.8	27	115	9.1	4	5	0.01
23	60	M	3.87	242	0.7	31	119	8	3	27	0.23
24	54	M	4	229	0.8	30	117	7.7	2.4	23	0.06
25	50	F	5.46	234	0.4	22	141	7.1	3.1	20	0.04
26	43	F	5.62	198	0.7	26	123	9	3.4	28	0.13
27	45	F	3.76	221	0.5	18	135	8.6	3.9	22	0.11
28	55	F	5.66	226	0.5	32	126	11.4	3.7	12	0.02
29	41	M	5.57	225	0.6	30	145	8.9	2.8	21	0.1
30	38	F	4.14	194	0.5	23	142	9.6	2.9	18	0.08

RESULTS

TABLE 1: GENDER WISE DISTRIBUTION OF STUDY GROUP AND CONTROL GROUP

Gender	GROUP			
	CASES		CONTROLS	
	Number	Percentage	Number	Percentage
Male	33	55.00	18	60.00
Female	27	45.00	12	40.00
Total	60	100	30	100
Chi-Square Value	0.20			
p-value	0.65			
Significant	Not Significant			

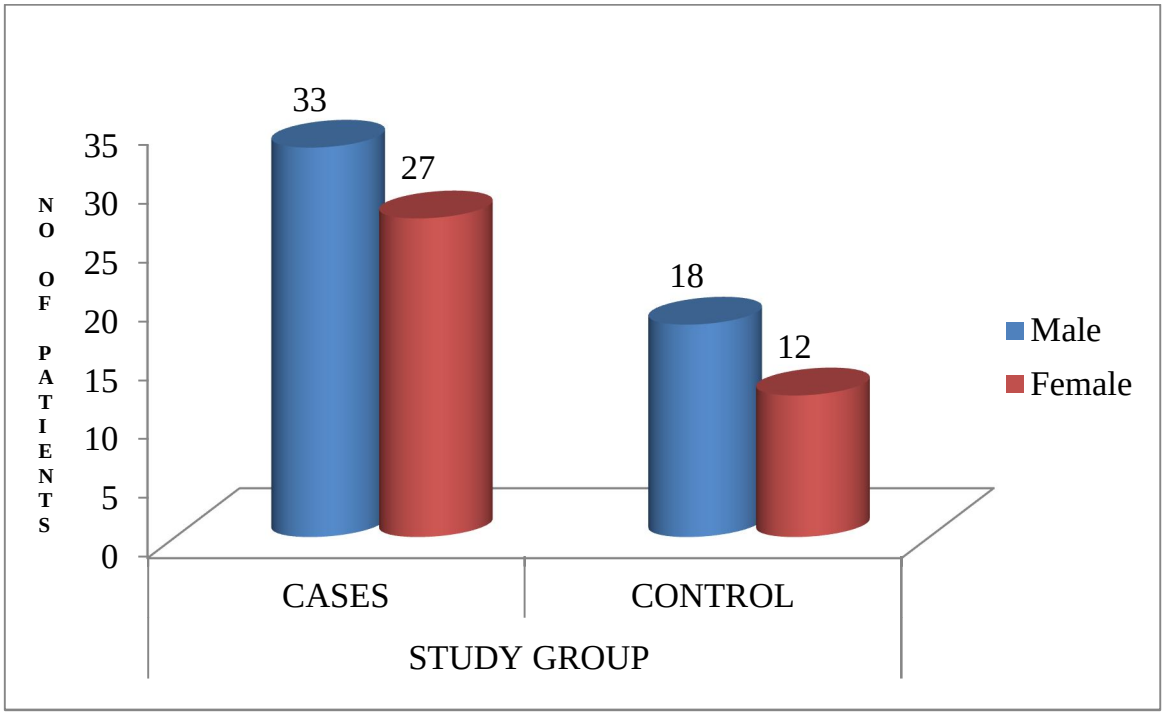


Table 1 and bar chart represent the gender wise distribution of cases and controls and the p value obtained was 0.65 not significant.

TABLE 2: MEAN AGE OF STUDY GROUP AND CONTROL GROUP

Age	GROUP	
	CASES	CONTROLS
Mean (Years)	51.67	52.53
SD	11.57	9.01
t-value	0.36	
p-value	0.72	
Significant	Not Significant	

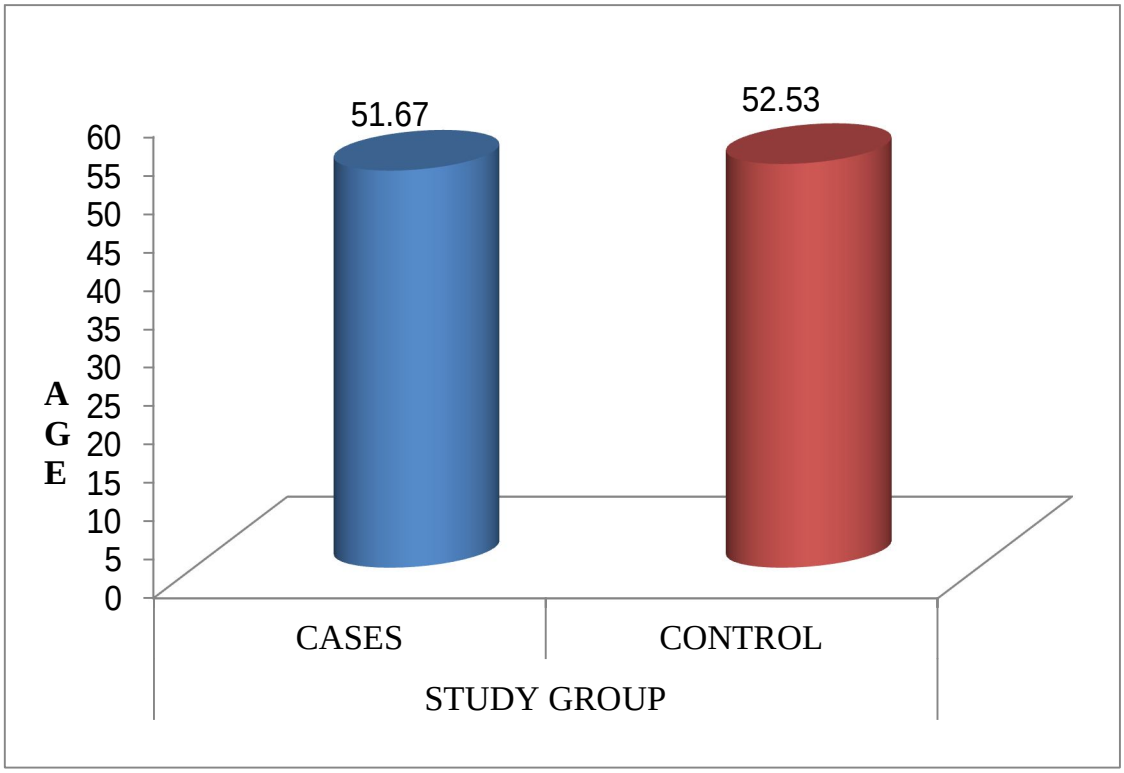


Table 2 and bar chart represent the mean age between the cases and controls, p value-0.72 was not significant

TABLE 3: KLOTHO BY STUDY GROUP AND CONTROL GROUP

KLOTHO	GROUP	
	CASES	CONTROLS
Mean	3.33	5.61
SD	1.37	2.12
t-value	6.18	
p-value	**0.001**	
Significant	Highly Significant	

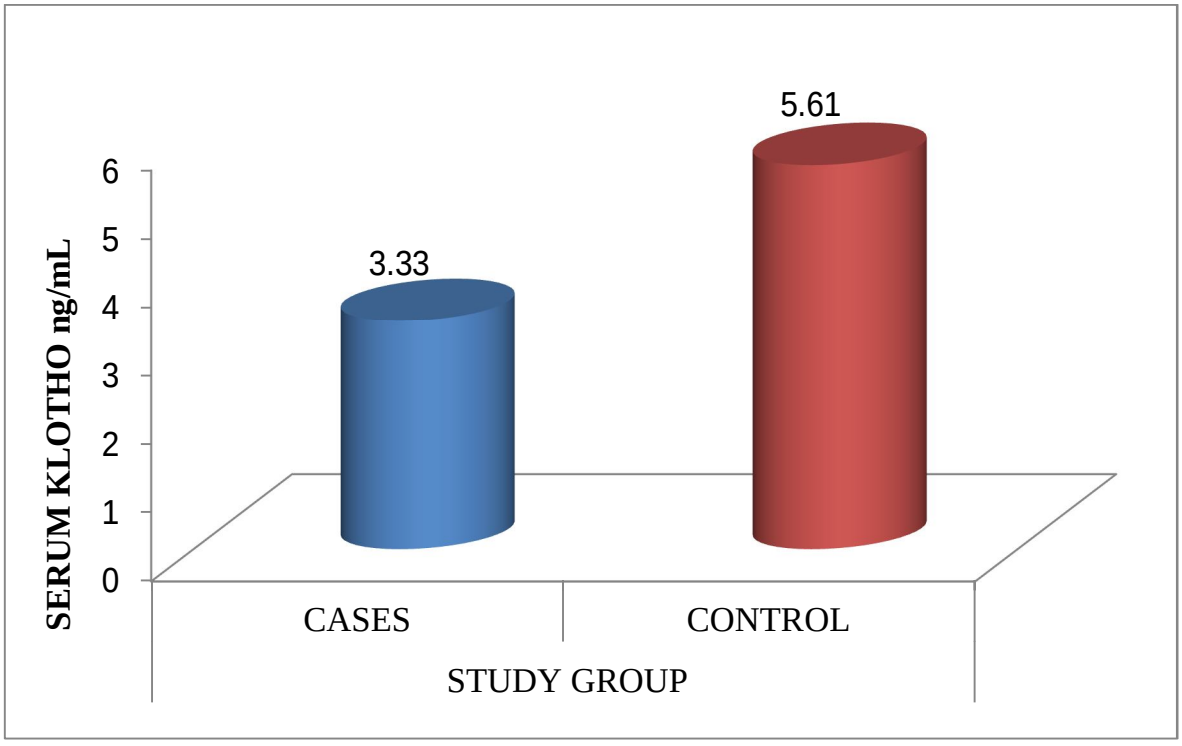
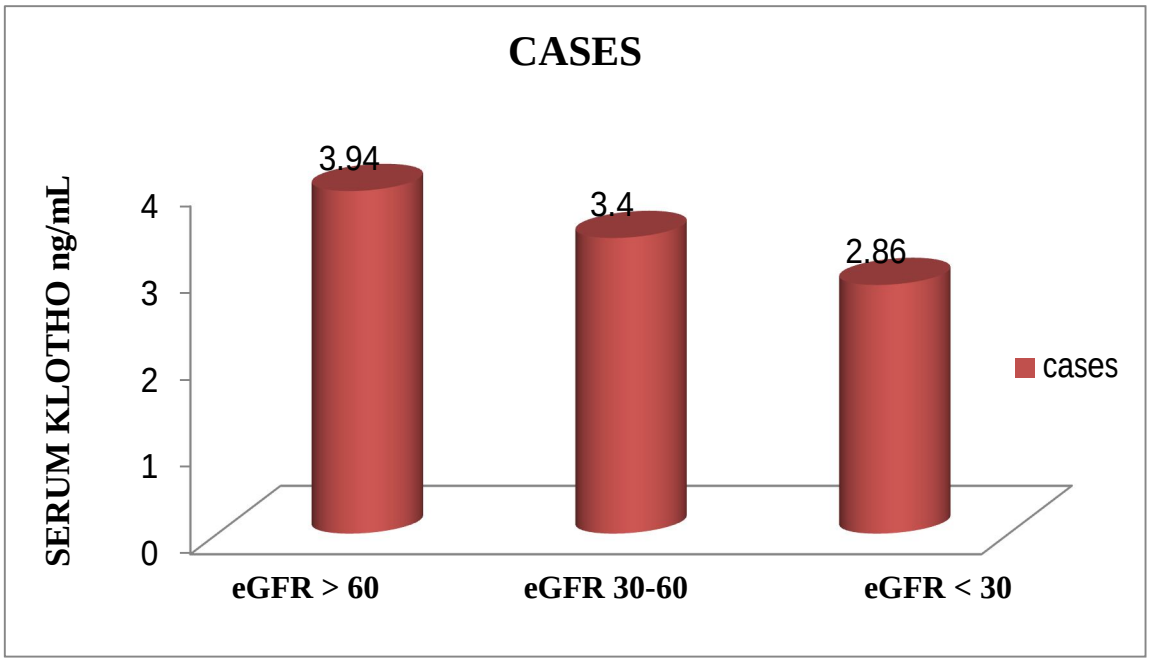


Table 3 and bar chart shows the comparison of Klotho between cases and controls. The mean Klotho level in control group- 5.61 ± 2.12 ng/mL and CKD cases – 3.33±1.37 ng/mL. A highly significant p value 0.001 was obtained.



The bar diagram compares serum Klotho levels between various eGFR categories in the CKD cases.

TABLE 4: FGF 23 BY STUDY GROUP AND CONTROL GROUP

FGF23	GROUP	
	CASES	CONTROLS
Mean	337.17	221.67
SD	156.17	37.13
t-value	3.98	
p-value	**0.001**	
Significant	Highly Significant	

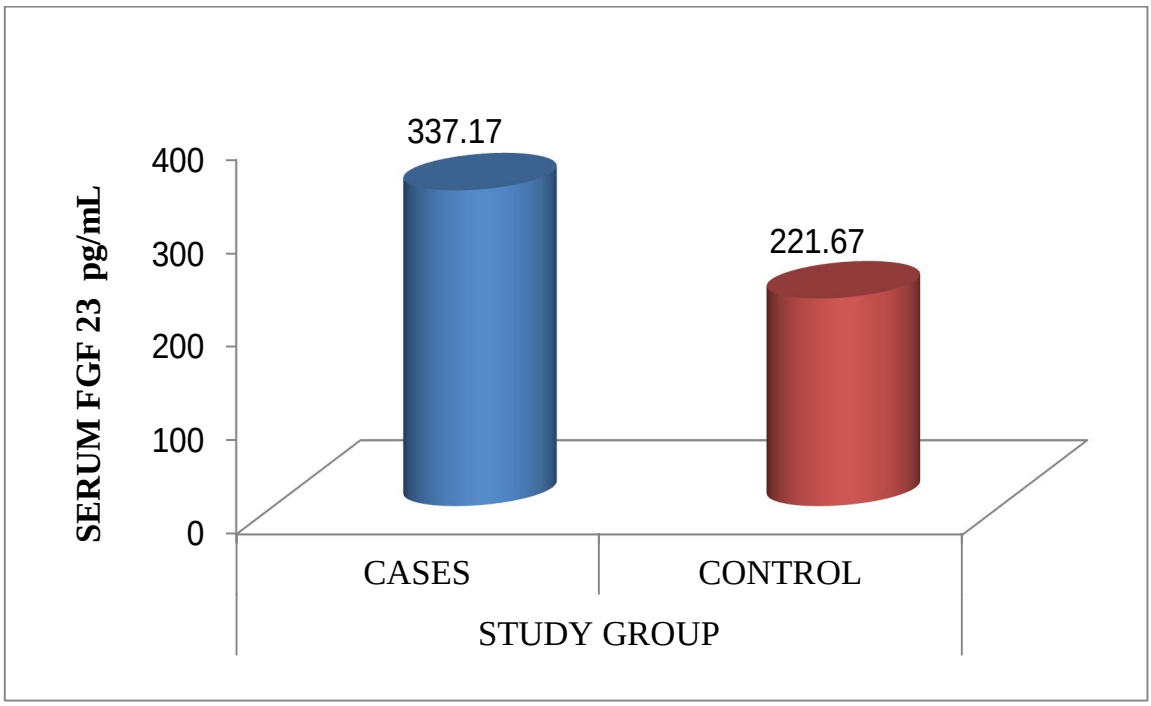
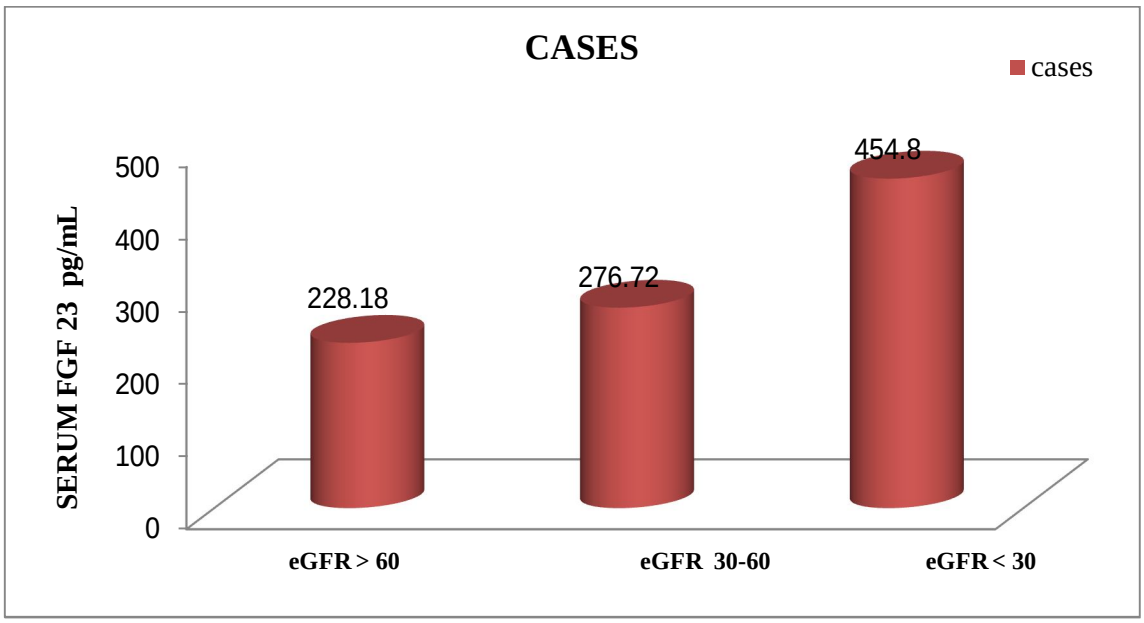


Table 5 shows the comparison of FGF23 between cases and control .The mean FGF23 in control group- 221.67±37.13 pg/mL and CKD cases- 337.17± 156.17 pg/mL. A highly significant P value 0.001 was obtained



The bar diagram compares serum FGF 23 levels between various eGFR categories in the CKD cases.

TABLE 5: CREATININEBY STUDY GROUP AND CONTROL GROUP

CRE FINAL	GROUP	
	CASES	CONTROLS
Mean	2.81	0.76
SD	2.25	0.19
t-value	4.97	
p-value	**0.001**	
Significant	Highly Significant	

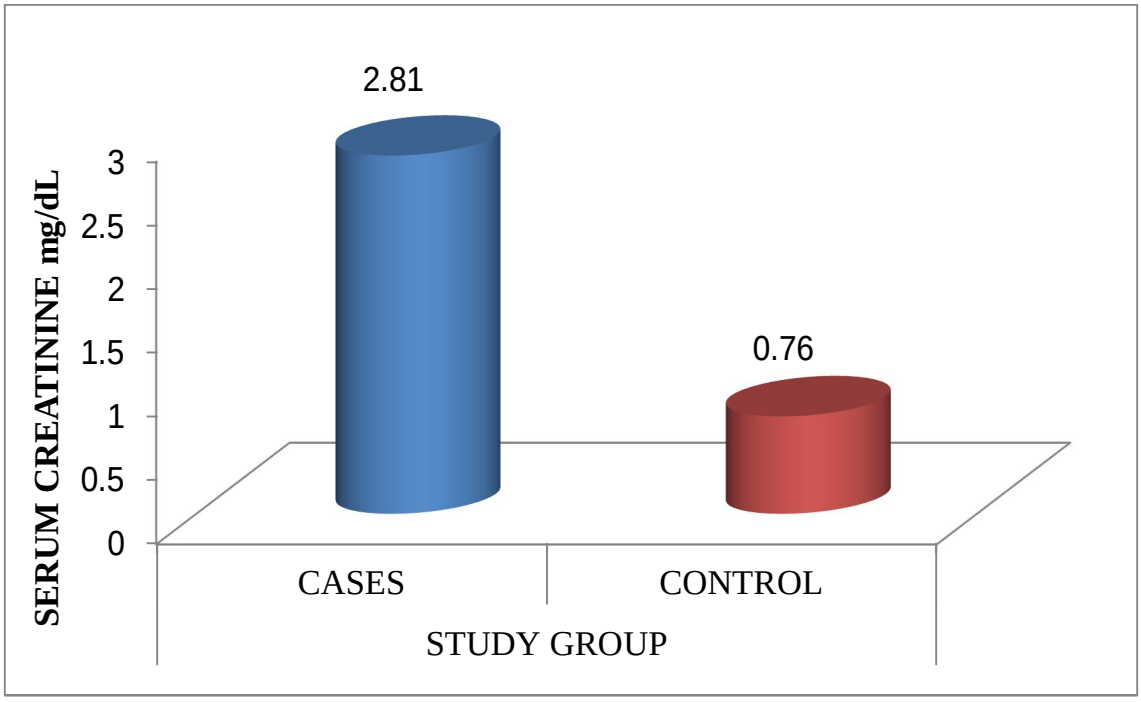


Table 5 and bar chart shows the comparison of creatinine between cases and control. The mean creatinine level in control group- 0.76± 0.19mg/dL and CKD cases - 2.81±2.25mg/dL. A highly significant p value 0.001 was obtained.

TABLE 6: UREA BY STUDY GROUP AND CONTROL GROUP

UREA	GROUP	
	CASES	CONTROLS
Mean	57.90	27.80
SD	33.51	7.48
t-value	4.85	
p-value	**0.001**	
Significant	Highly Significant	

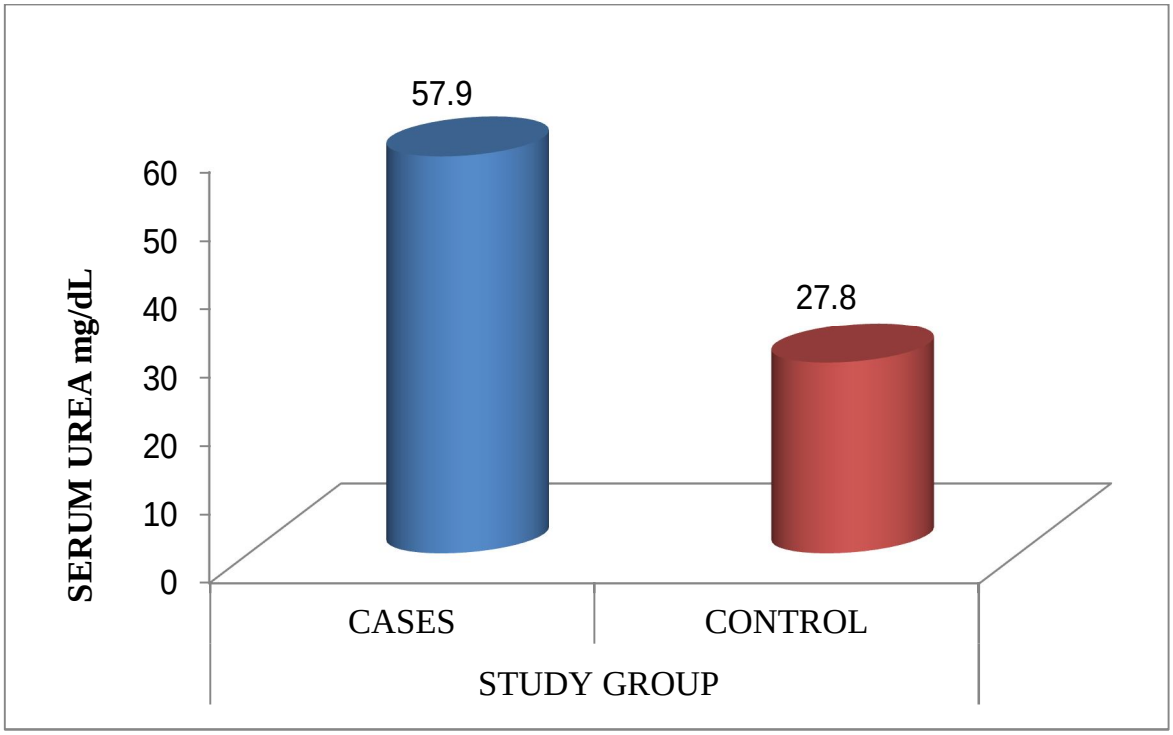


Table 6 and bar chart shows the comparison of urea between cases and control. The mean urea level in control group- 27.8± 7.48mg/dL and CKD cases – 57.9±33.51mg/dL. A highly significant p value 0.001 was obtained.

TABLE 7: CALCIUM BY STUDY GROUP AND CONTROL GROUP

CALCIUM	GROUP	
	CASES	CONTROLS
Mean	8.34	9.33
SD	1.03	0.83
t-value	4.57	
p-value	**0.001**	
Significant	Highly Significant	

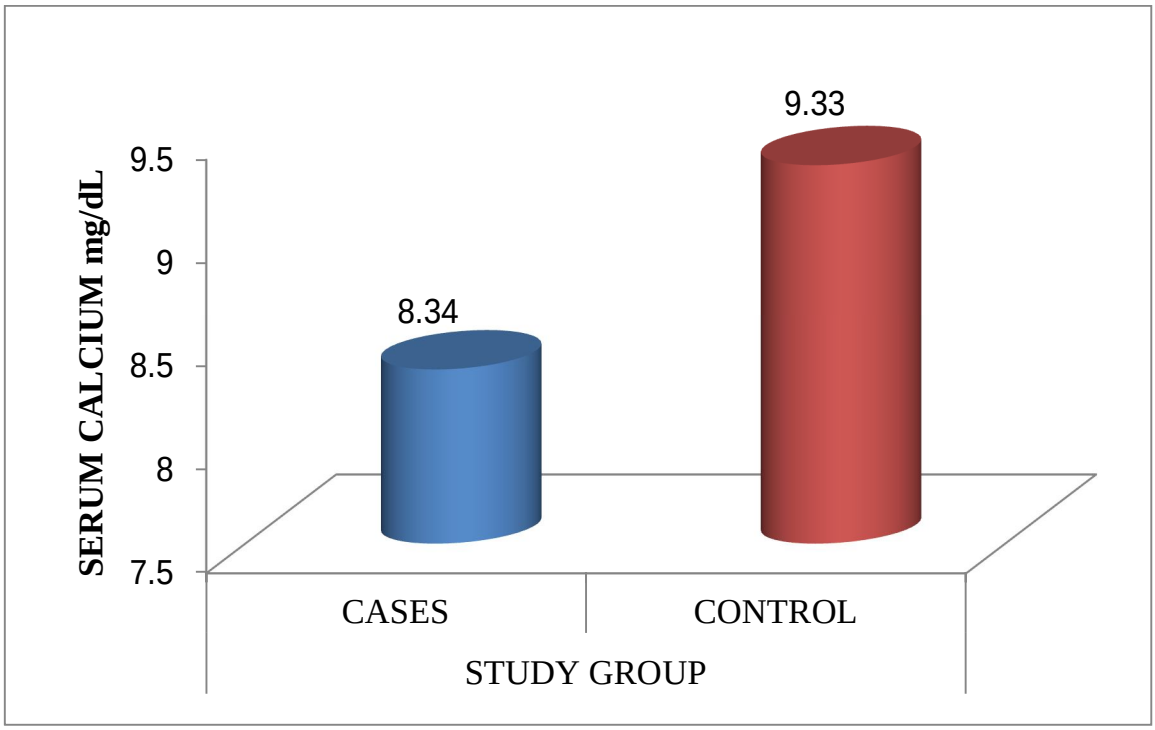


Table 7 and bar chart shows the comparison of calcium between cases and control. The mean calcium level in control group- 9.33± 0.83mg/dL and CKD cases –8.34±1.03mg/dL. A highly significant p value 0.001 was obtained.

TABLE 8: PHOSPHATE BY STUDY GROUP AND CONTROL GROUP

PHOSPHATE	GROUP	
	CASES	CONTROLS
Mean	3.66	3.50
SD	1.14	0.46
t-value	0.74	
p-value	0.46	
Significant	Not Significant	

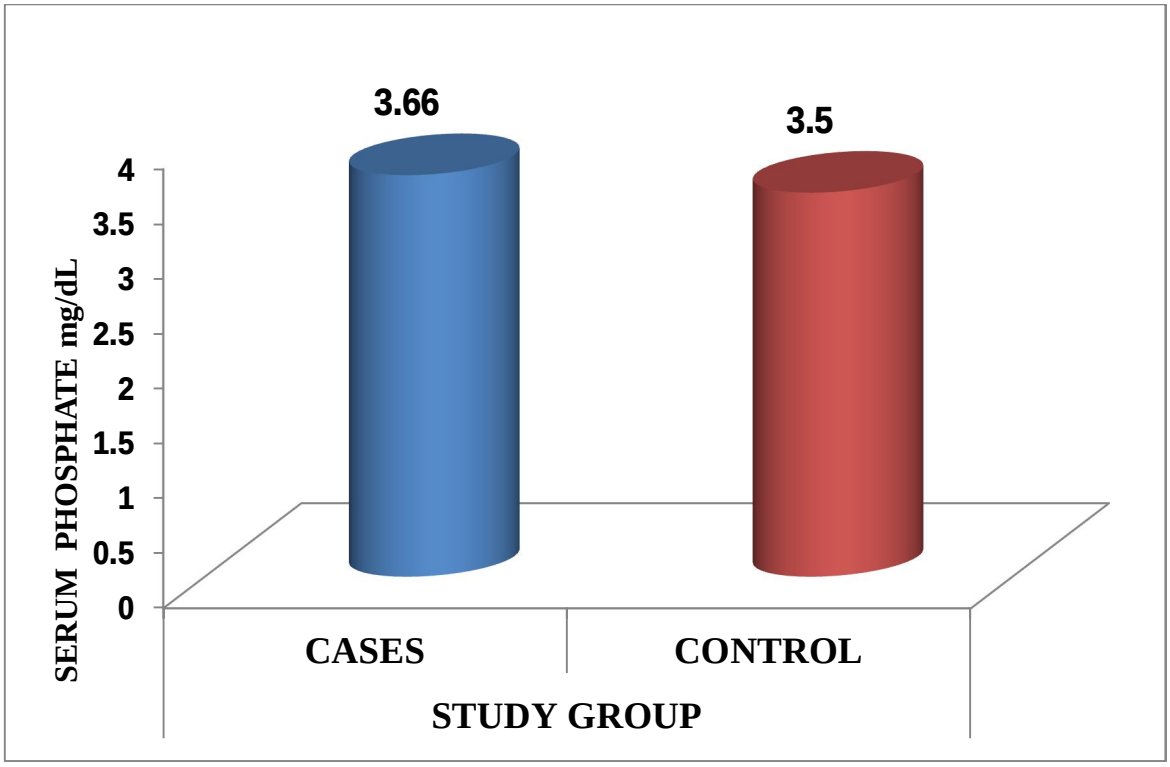


Table 8 and bar chart shows the comparison of phosphate between cases and control. The mean phosphate level in control group- 3.5± 0.46mg/dL and CKD cases –3.66±1.14mg/dL. p value obtained is 0.46 which is not significant.

TABLE 9: PCR BY STUDY GROUP AND CONTROL GROUP

	GROUP	
	CASES	**CONTROL**
Mean	2.17	0.09
SD	2.89	0.05
t-value	3.95	
p-value	**0.001**	
Significant	Highly Significant	

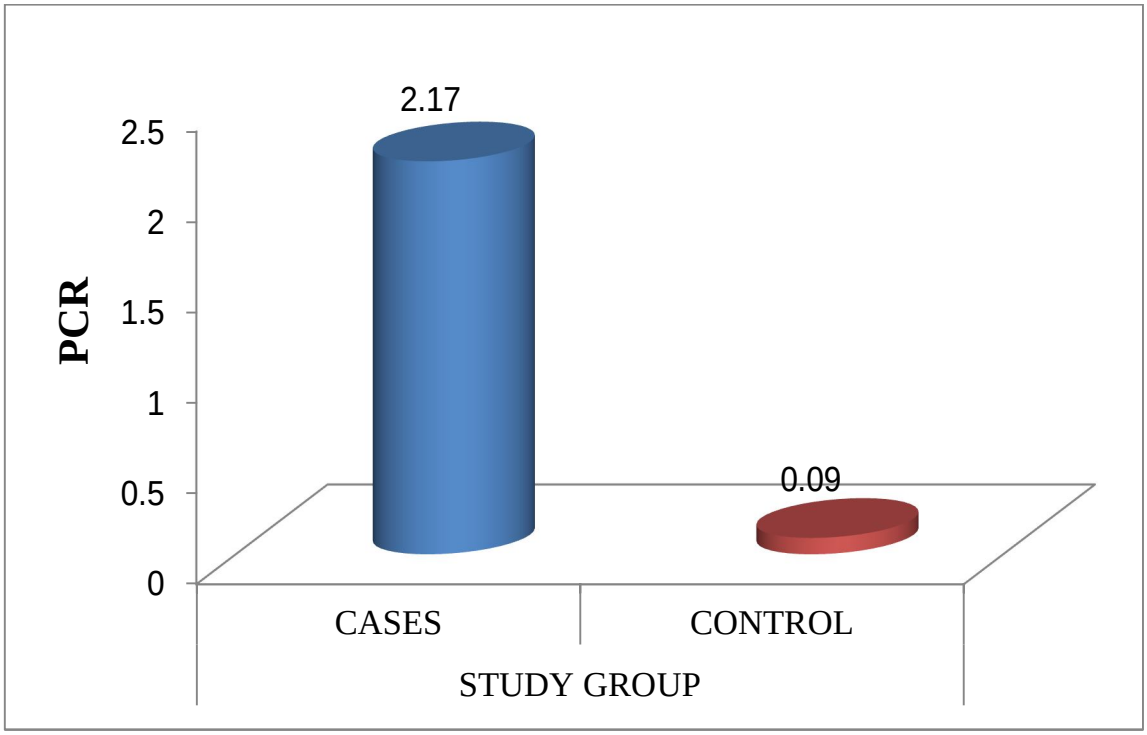


Table 7 and bar chart shows the comparison of protein creatinine ratio between cases and control. The mean PCR in control group- 0.09± 0.05 and CKD cases –2.17±2.89. A highly significant p value 0.001 was obtained.

TABLE 10: PTH BY STUDY GROUP AND CONTROL GROUP

PTH	GROUP	
	CASES	CONTROLS
Mean	90.15	25.50
SD	112.95	12.02
t-value	3.12	
p-value	**0.002**	
Significant	Highly Significant	

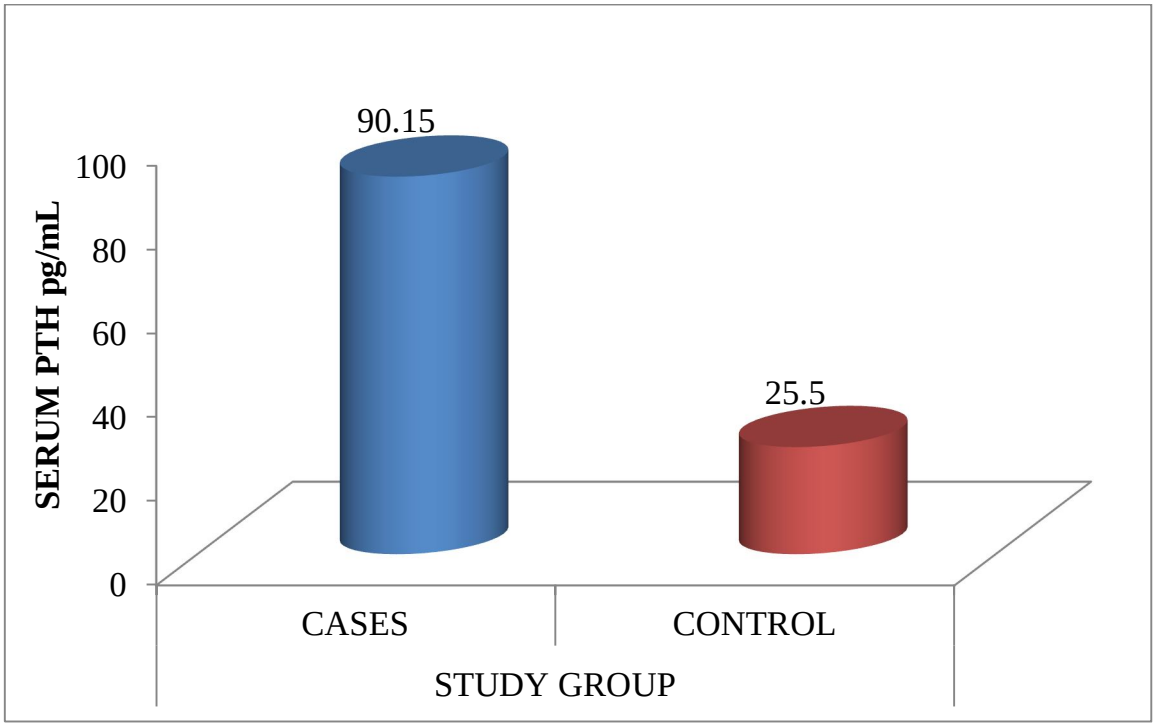


Table 10 and bar chart shows the comparison of parathyroid hormone levels between cases and control. The mean PTH in control group- 25.5pg/mL and CKD cases –90.15pg/mL. A highly significant p value 0.001 was obtained.

TABLE 11: DIAGNOSIS

SLNO	Diagnosis	Number	Percentage
1	ANALGESIC ABUSE	1	1.67
2	ATN / CKD /POISONING	1	1.67
3	CIN	2	3.33
4	DM	9	15.00
5	DM /CAD	1	1.67
6	DM / FSGS	1	1.67
7	DM /HYPOTHYROIDISM	2	3.33
8	DM / SHT	21	35.00
9	DM / SHT / IGA	1	1.67
10	HT / CIN / MN	1	1.67
11	IGA NEP	5	8.32
12	PT	1	1.67
13	SHT	11	18.32
14	SHT /CIN	1	1.67
15	SHT / SNAKE BITE	1	1.67
16	YOUNG STROKE / HT	1	1.67
	TOTAL	**60**	**100**

Table 11 shows the various etiology for chronic kidney disease in the study population.

Table 12:

Correlation between Klotho and eGFR (cases)

r (correlation)	**0.22**
Significant at	**0.05**

Correlation between Klotho and eGFR (controls)

r (correlation)	**0.03**
Significant at	**-**

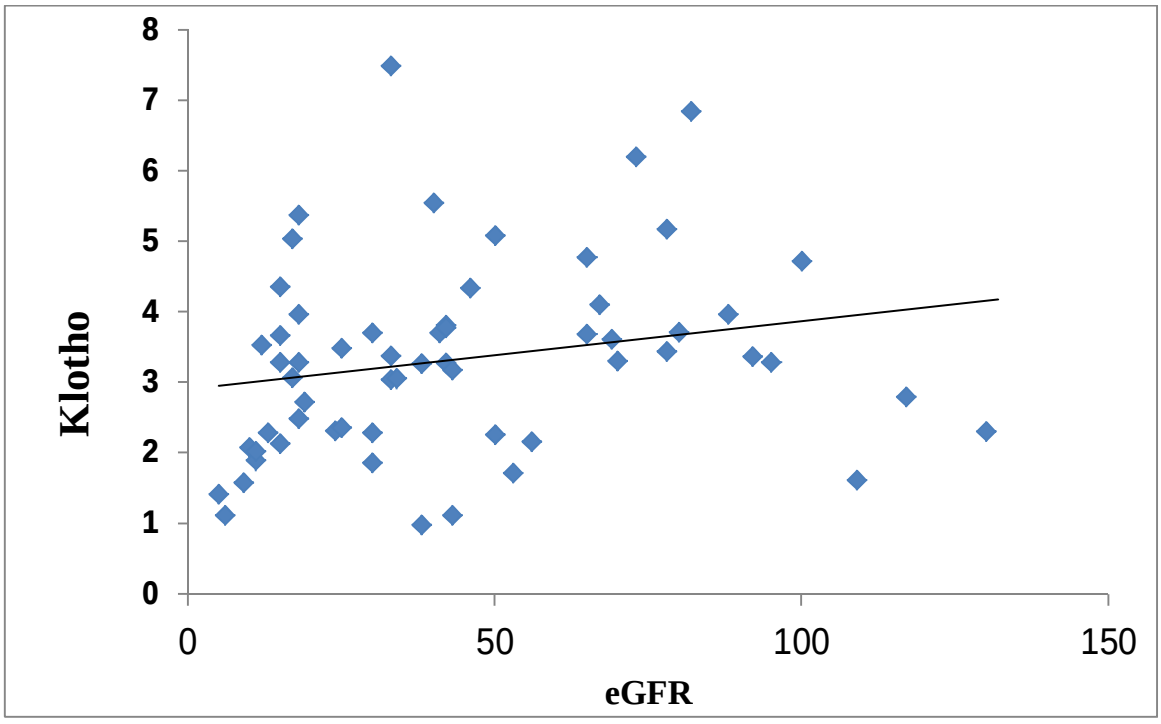


Table12 and figure show the correlation between Klotho and eGFR. R value is 0.22 and Klotho and eGFR are positively correlated.

Table 13:
Correlation between eGFR and FGF23 (cases)

r (correlation)	**-0.58**
Significant at	**0.001**

Correlation between eGFR and FGF23 (controls)

r (correlation)	**-0.24**
Significant at	**-**

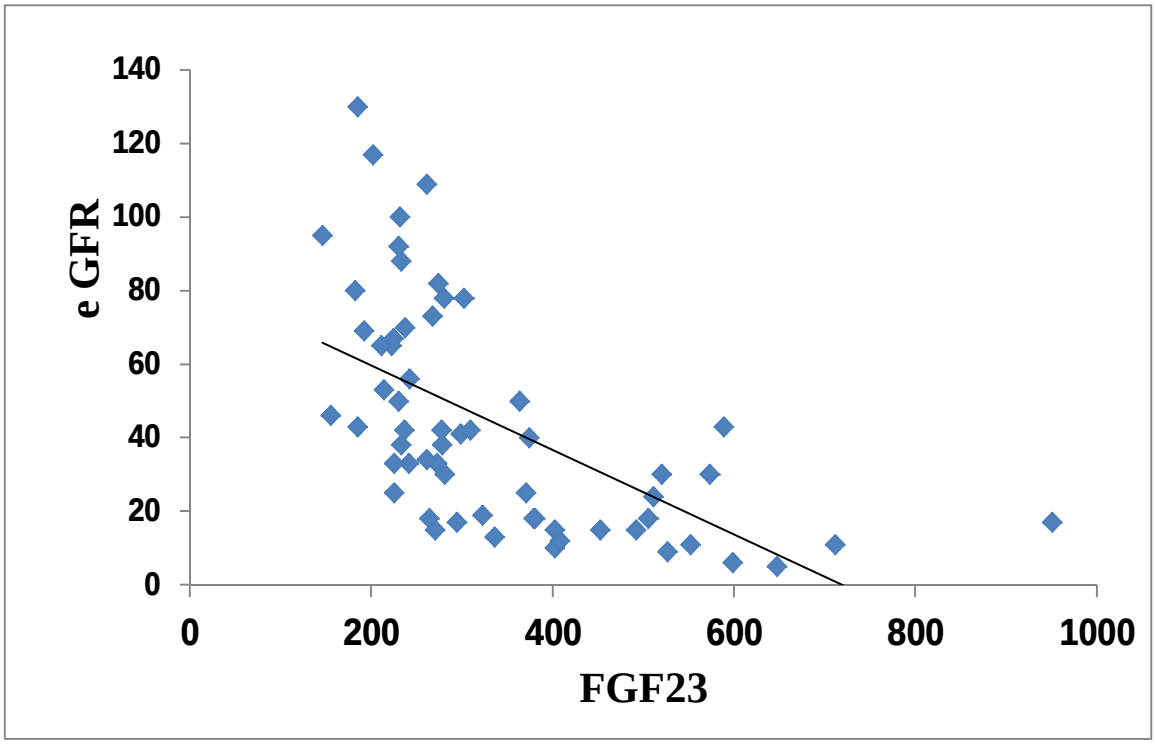


Table13 and figure shows the correlation between FGF 23 and eGFR. R value is –0.58and FGF 23 and eGFR are negatively correlated.

Table 14:

Correlation between Klotho and FGF23 (Cases)

r (correlation)	**-0.40**
Significant at	**0.002**

Correlation between Klotho and FGF23 (Control)

r (correlation)	**-0.47**
Significant at	**0.01**

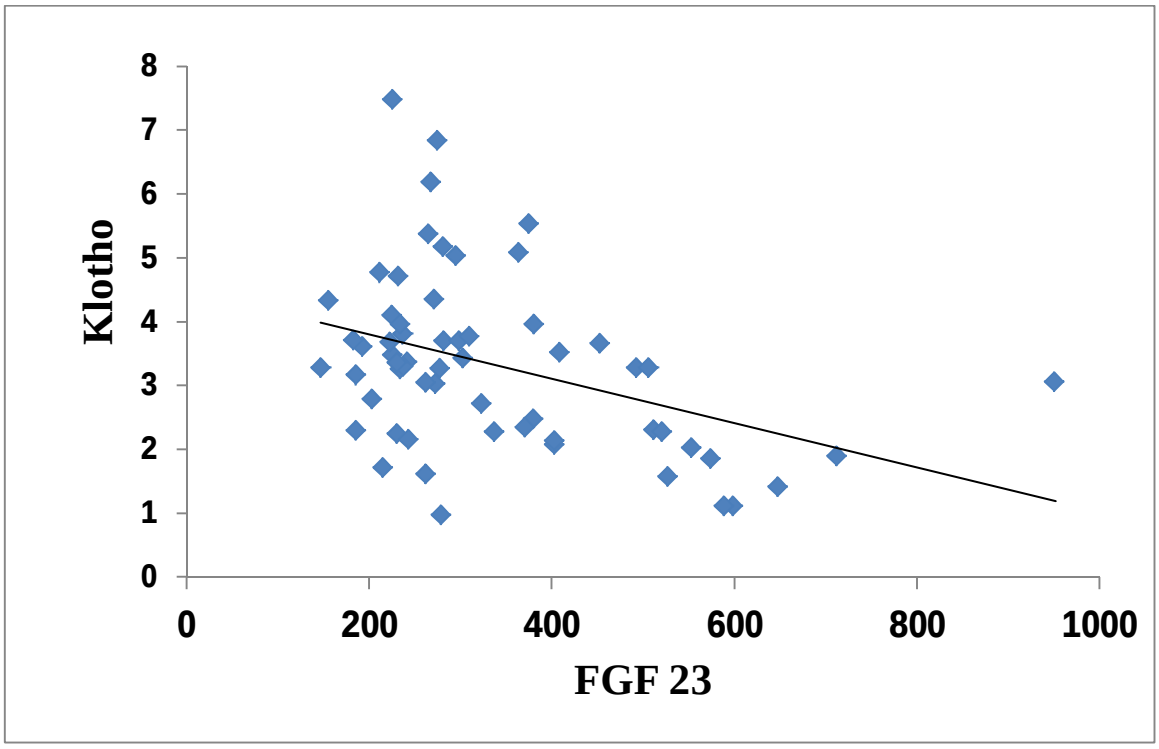
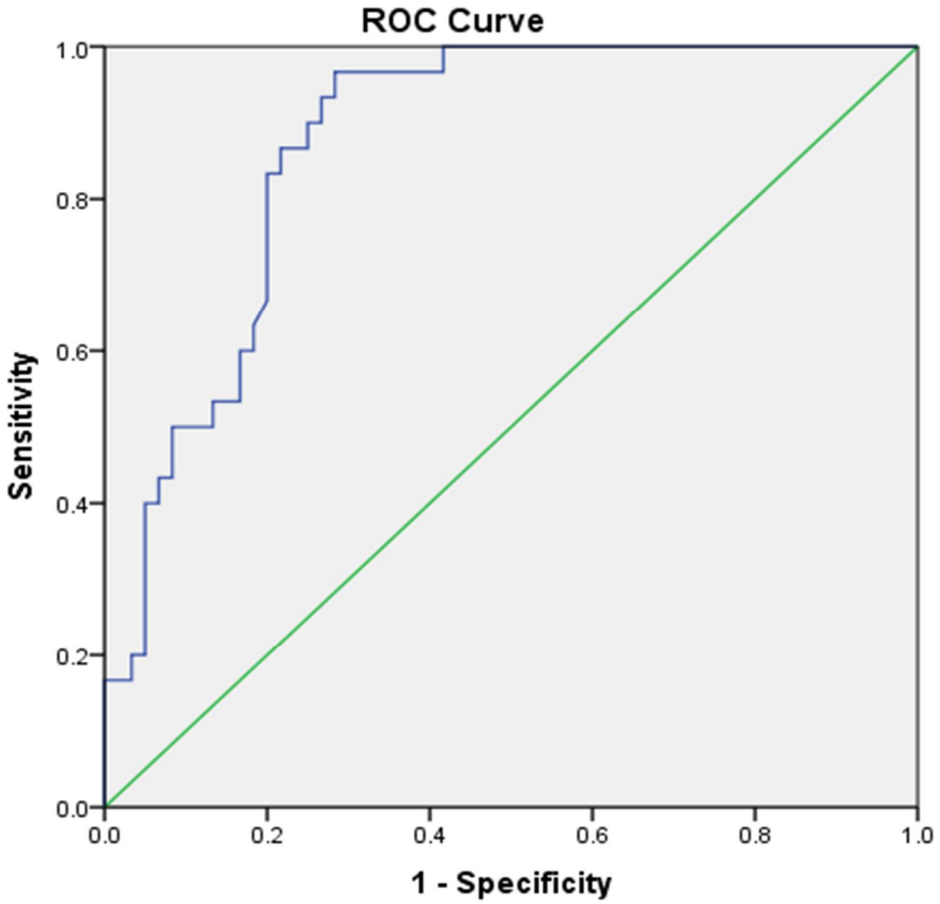
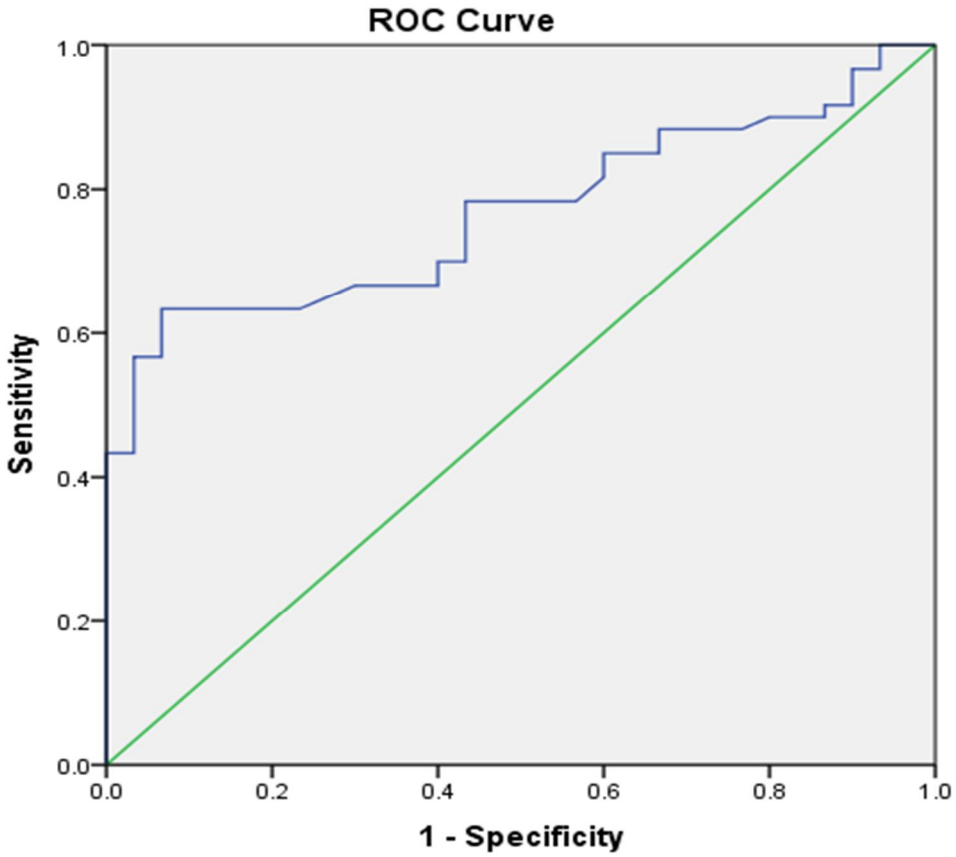


Table 14 and figure shows the correlation between Klotho and FGF23. R value is –0.40 and Klotho and FGF 23 are negatively correlated.



Area Under the Curve				
Test Result Variable(s): Klotho				
Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
.872	.036	.000	.801	.942
Cut off value : 3.74				



Diagonal segments are produced by ties.

Area Under the Curve				
Test Result Variable(s): FGF 23				
Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
0.768	.048	.000	.673	.862
Cut off:260.5				

Regression table

Model Summary				
Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.804[a]	.646	.611	27.047
a. Predictors: (Constant), PTH, PCR, Klotho, Phosphate, Calcium, Creatinine, FGF 23, Urea				

coefficients[a]						
Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
1	(Constant)	89.333	33.034		2.704	.008
	KLOTHO	3.419	1.743	.155	1.962	.053
	FGF23	-.034	.037	-.109	-.913	.364
	CREATININE	-6.447	3.149	-.309	-2.047	.044
	UREA	-.473	.227	-.339	-2.083	.040
	CALCIUM	-1.110	3.698	-.028	-.300	.765
	PHOSPHATE	7.149	3.924	.160	1.822	.072
	PCR	-2.478	1.214	-.146	-2.041	.044
	PTH	-.025	.052	-.056	-.478	.634
a. Dependent Variable: eGFR						

Discussion

DISCUSSION

The present study aims to correlate serum soluble α Klotho and FGF23 levels in chronic kidney disease as they have important roles in the pathogenesis of CKD-MBD and predictive markers for CKD progression.

In the present study we recruited people in two groups

Group 1: Chronic kidney disease cases

Group 2: Controls

From tables 1 and 2 there is no significant difference between the groups with respect to age and sex.

The mean Klotho levels in control group are 5.61 ± 2.12 ng/mL and CKD cases – 3.33 ± 1.37 ng/mL. A highly significant p value of 0.001 was obtained.

The mean FGF 23 in control group- 221.67 ± 37.13 pg/mL and CKD cases- 337.17 ± 156.17 pg/mL. A highly significant p value of 0.001 was obtained.

In concordance with experimental models of CKD and with papers in the literature our patients showed strikingly reduced serum soluble α-Klotho levels and considerably increased FGF 23 levels as compared to reference control[77].

Likewise our mean values of Klotho and FGF23 in CKD and in normal controls are comparable to those in the literature that employ our same method of assay[78].

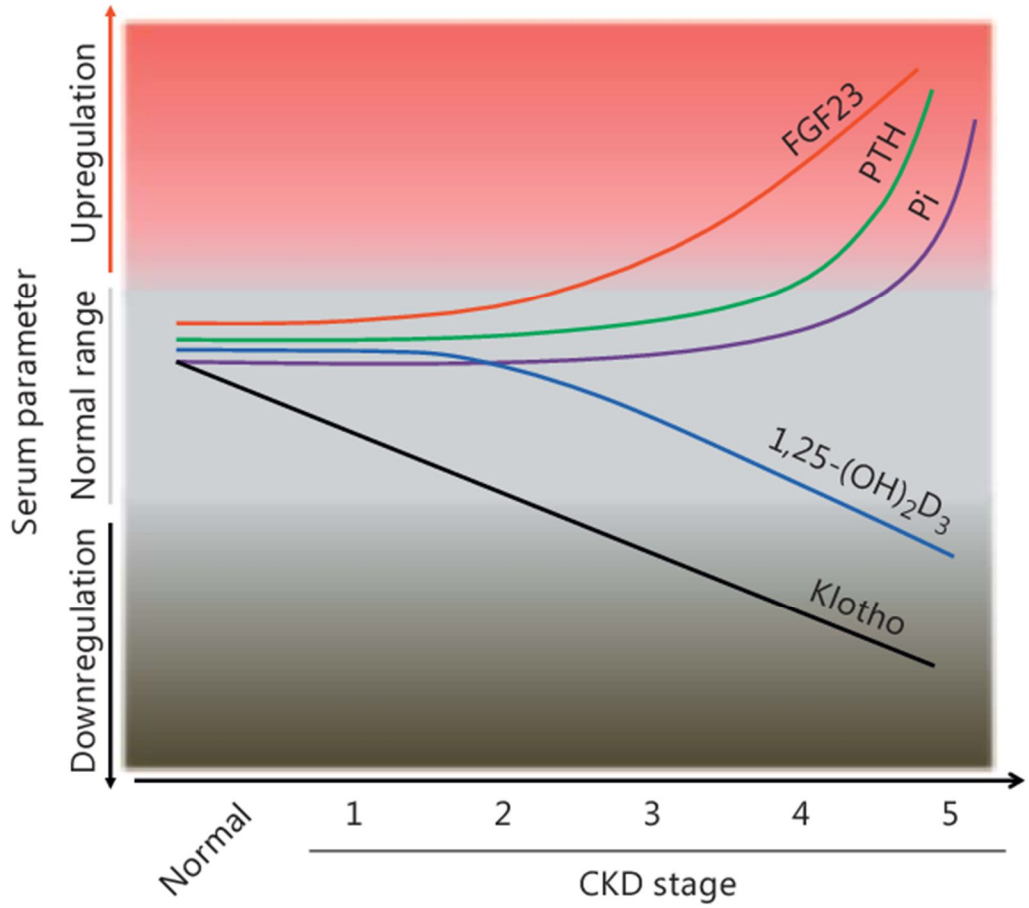


Figure 8: Changes in serum parameters during progression of chronic kidney disease.

Courtesy: John et al. Am J Kidney Dis.2011 July; 58(1):127-134

Correlation between Klotho and eGFR was done and they were positively correlated with r value of 0.22.

Correlation between FGF23 and eGFR was done and they were negatively correlated with r value of -0.58

Correlation between Klotho and FGF23 was done and they are negatively correlated with r value of -0.40.

Renal function negatively afflicted s-Klotho levels with detectable reduction starting from CKD stage 2. Since s-Klotho is usually excreted via urine, this reduction in serum levels along with progression of renal damage can be explained by diminished renal synthesis.

“In case of stable renal Klotho production a reduced excretion due to renal damage would increase serum levels. Alternatively if renal excretion persisted to be normal, serum levels would not decrease considerably. On the contrary increased renal excretion seems highly improbable since there is available clinical and experimental data of reduced urinary s-Klotho in CKD”.

Serum levels of FGF 23 were elevated in CKD population on comparing with healthy individuals, with increments detectable since CKD stage 2. Correlations of FGF 23 were negative with eGFR. These data suggest that bone cells somehow detect the reduction of GFR very early and increase FGF 23 synthesis, chiefly to decrease vitamin D synthesis and to maintain phosphate concentration within normal range.

The consensual increase of serum phosphate, PTH and FGF 23 in CKD individuals can be secondary to the reduction of renal function.

In the present study a negative relationship emerged between s-Klotho and FGF 23 .This supports the hypothesis that s-Klotho whose levels in circulation are highly associated to renal function are most likely secondary to reduced expression of trans membrane Klotho, which can be considered as a sensitive

marker of trans membrane Klotho expression. Thus it explains early development of tubular resistance to FGF 23. The synthesis ofFGF23 in a state of hyperphosphatemia increases considerably in the bone.

A recent literature with histological data from patients with glomerulonephritis showed parallel reduction of renal Klotho and of s-Klotho together with increments in FGF 23, which is in agreement with our data[79].

Transgenic animals selectively null for renal Klotho have negligible shedding of Klotho from renal explants and circulating levels reduced by 80%, thus revealing the kidney as a major contributor of circulating Klotho[23].

FGF 23 is not just a marker of the derangements of calcium – phosphate metabolism in CKD but a causative factor for secondary hyperparathyroidism and for cardiovascular mortality and morbidity.

FGF 23 may reveal information about phosphate related toxicity that cannot be obtained by measurements of serum phosphate.

FGF 23 could represent a promising therapeutic target that might improve the prognosis of CKD.

In our study serum Klotho and FGF 23 levels have started showing significant changes from early stages of CKD but with substantially normal values of calcium and phosphate. The significant changes in calcium and phosphate were

seen only in late stage of CKD. Similarly significant changes in PTH were observed from stage III of CKD.

This confirms that changes in serum calcium and phosphate are not reliable to detect early CKD-MBD while s-Klotho and FGF23 seem more sensitive than PTH and vitamin D.

From table 11, the commonest etiology predisposing to CKD in our population was found to be diabetes mellitus and hypertension.

To evaluate the operating characteristics of Klotho and FGF23 as a prognostic tool for the progression of kidney disease we performed receiver operating characteristic curve analysis for Klotho and FGF 23.

For serum soluble α-Klotho the area under the curve is 0.872 at 95% confidence interval and the cut off level is 3.74 with sensitivity of 87% and specificity of 83%.

For FGF 23 the area under the curve is 0.768 at 95% confidence interval and the cut off is 260.5 with sensitivity of 76% and specificity of 80%.

Multiple regression analysis was performed to evaluate relationship between eGFR and other parameters (Klotho, FGF23, creatinine, urea, PTH, calcium, phosphate and urine PCR). Even though Klotho and FGF 23 were not independent predictors for chronic kidney disease they have good correlation with disease progression.

Conclusion

CONCLUSION

The study of serum soluble α Klotho and FGF 23 levels in chronic kidney disease has been undertaken to unravel the various linking wires to highlight the values and establish the role of the interlinked novel markers.

Several analytical procedures have been put forth while tabulating the results of the various parameters of this study along with the relevant statistical evaluation.

Therefore, we conclude that

- Serum soluble α-Klotho decreases in chronic kidney disease.
- Fibroblast growth factor 23 increases in chronic kidney disease.
- There is a significant positive correlation between Klotho and eGFR.
- There is a significant negative correlation between FGF 23 and eGFR.
- There is a significant negative correlation between Klotho and FGF 23.
- Changes in Klotho and FGF 23 precede the changes in PTH, vitamin D, calcium and phosphate.

Therefore Klotho and FGF 23 may be used as prognostic markers of CKD progression and complications.

Limitations of the Study

LIMITATIONS OF THE STUDY

Patients with early stages of CKD are included in the study more than those with later stages to prove it as an early biomarker.

Similarly patients on hemodialysis and who were on renal transplant were not included in the study.

e GFR is calculated using creatinine based equation. It would be better to measure GFR using inulin clearance.

Scope for Further Studies

FURTHER SCOPE OF THE STUDY

FGF23 functions as a circulating factor that can directly attribute to cardiac hypertrophy, inflammation and impaired host response in CKD. Further studies can be done to assess the Klotho and FGF 23 levels in CKD associated complications.

Novel analytical methods have to be developed to estimate Klotho and FGF 23 both in vitro and in vivo

Widening the knowledge of FGF 23 –FGFR biology will ultimately lead to the discovery of novel drug targets and the advancement of pharmacological interventions that reduce the burden of cardiovascular complications, decrease systemic inflammation, prevent infections and prolong kidney transplant survival and ultimately decreased mortality.

Further clinical studies could be done to emphasize the potential differences in gender, ethnicity and genetic characteristics.

Bibliography

BIBLIOGRAPHY

1. Cozzolino M, Ureña-Torres P, Vervloet MG, Brandenburg V, Bover J, Goldsmith D, et al. Is chronic kidney disease-mineral bone disorder (CKD-MBD) really a syndrome? Nephrol Dial Transplant. 2014 Oct 1;29(10):1815–20.

2. KISU_v7_i1_COVER.indd. Kidney Int Suppl. 2017;60.

3. National Kidney Foundation. Kidney Disease. New York, NY: National Kidney Foundation:2008. Available at http://www.kidney .org/kidney disease

4. Matovinovi MS. 1. PATHOPHYSIOLOGY AND CLASSIFICATION OF KIDNEY DISEASES. :10.

5. EMT and proteinuria as progression factors - Kidney International [Internet]. [cited 2018 Sep 10]. Available from: https://www.kidney-international.org/article/S0085-2538(15)53723-X/fulltext

6. Abbate M, Zoja C, Remuzzi G. How Does Proteinuria Cause Progressive Renal Damage? J Am Soc Nephrol. 2006 Nov 1;17(11):2974–84.

7. Ito S, Fujimori T, Hayashizaki Y, Nabeshima Y. Identification of a novel mouse membrane-bound family 1 glycosidase-like protein, which carries an atypical active site structure. Biochim Biophys Acta. 2002 Jul 19;1576(3):341–5.

8. Ito S, Kinoshita S, Shiraishi N, Nakagawa S, Sekine S, Fujimori T, et al. Molecular cloning and expression analyses of mouse betaklotho, which encodes a novel Klotho family protein. Mech Dev. 2000 Nov;98(1–2):115–9.

9. Klotho (biology) - an overview | ScienceDirect Topics [Internet]. [cited 2018 Sep 28]. Available from: https://www.sciencedirect.com/topics/medicine-and-dentistry/klotho-biology

10. Hu MC, Kuro-o M, Moe OW. Klotho and Chronic Kidney Disease. In: Razzaque MS, editor. Contributions to Nephrology [Internet]. Basel: S. KARGER AG; 2013

[cited 2018 Jun 12]. p. 47–63. Available from: https://www.karger.com/Article/FullText/346778

11. Hu MC, Kuro-o M, Moe OW. Klotho and Chronic Kidney Disease. In: Razzaque MS, editor. Contributions to Nephrology [Internet]. Basel: S. KARGER AG; 2013 [cited 2018 Jun 21]. p. 47–63. Available from: https://www.karger.com/Article/FullText/346778

12. Cha SK, Ortega B, Kurosu H, Rosenblatt KP, Kuro-o M, Huang CL. Removal of sialic acid involving Klotho causes cell-surface retention of TRPV5 channel via binding to galectin-1. Proc Natl Acad Sci U S A. 2008 Jul 15;105(28):9805–10.

13. Chang Q, Hoefs S, Kemp AW van der, Topala CN, Bindels RJ, Hoenderop JG. The ß-Glucuronidase Klotho Hydrolyzes and Activates the TRPV5 Channel. Science. 2005 Oct 21;310(5747):490–3.

14. Zhao Y, Banerjee S, Dey N, LeJeune WS, Sarkar PS, Brobey R, et al. Klotho Depletion Contributes to Increased Inflammation in Kidney of the db/db Mouse Model of Diabetes via RelA (Serine)536 Phosphorylation. Diabetes. 2011 Jul;60(7):1907–16.

15. Lindberg K, Amin R, Moe OW, Hu M-C, Erben RG, Östman Wernerson A, et al. The Kidney Is the Principal Organ Mediating Klotho Effects. J Am Soc Nephrol JASN. 2014 Oct;25(10):2169–75.

16. Hu MC, Shi M, Zhang J, Addo T, Cho HJ, Barker SL, et al. Renal Production, Uptake, and Handling of Circulating αKlotho. J Am Soc Nephrol JASN. 2016 Jan;27(1):79–90.

17. Hu M-C, Shi M, Zhang J, Quiñones H, Kuro-o M, Moe OW. Klotho deficiency is an early biomarker of renal ischemia–reperfusion injury and its replacement is protective. Kidney Int. 2010 Dec;78(12):1240–51.

18. Akimoto T, Yoshizawa H, Watanabe Y, Numata A, Yamazaki T, Takeshima E, et al. Characteristics of urinary and serum soluble Klotho protein in patients with different degrees of chronic kidney disease. BMC Nephrol. 2012 Nov 23;13(1):155.

19. Rotondi S, Pasquali M, Tartaglione L, Muci ML, Mandanici G, Leonangeli C, et al. Soluble α -Klotho Serum Levels in Chronic Kidney Disease. Int J Endocrinol. 2015;2015:872193.

20. Chen C-D, Tung TY, Liang J, Zeldich E, Tucker Zhou TB, Turk BE, et al. Identification of cleavage sites leading to the shed form of the anti-aging protein klotho. Biochemistry. 2014 Sep 2;53(34):5579–87.

21. Hu MC, Fan L, Crowder LA, Karim-Jimenez Z, Murer H, Moe OW. Dopamine acutely stimulates Na+/H+ exchanger (NHE3) endocytosis via clathrin-coated vesicles: dependence on protein kinase A-mediated NHE3 phosphorylation. J Biol Chem. 2001 Jul 20;276(29):26906–15.

22. Aging-related kidney damage is associated with a decrease in klotho expression and an increase in superoxide production [Internet]. [cited 2018 Sep 10]. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3168600/

23. Hu MC, Kuro-o M, Moe OW. Secreted Klotho and Chronic Kidney Disease. In: Kuro-o M, editor. Endocrine FGFs and Klothos [Internet]. New York, NY: Springer US; 2012 [cited 2018 Jun 12]. p. 126–57. Available from: http://link.springer.com/10.1007/978-1-4614-0887-1_9

24. Haruna Y, Kashihara N, Satoh M, Tomita N, Namikoshi T, Sasaki T, et al. Amelioration of progressive renal injury by genetic manipulation of Klotho gene. Proc Natl Acad Sci U S A. 2007 Feb 13;104(7):2331–6.

25. Kendrick J, Chonchol M. The Role of Phosphorus in the Development and Progression of Vascular Calcification. Am J Kidney Dis Off J Natl Kidney Found. 2011 Nov;58(5):826–34.

26. Morishita K, Shirai A, Kubota M, Katakura Y, Nabeshima Y, Takeshige K, et al. The progression of aging in klotho mutant mice can be modified by dietary phosphorus and zinc. J Nutr. 2001 Dec;131(12):3182–8.

27. Liu S, Tang W, Zhou J, Stubbs JR, Luo Q, Pi M, et al. Fibroblast growth factor 23 is a counter-regulatory phosphaturic hormone for vitamin D. J Am Soc Nephrol JASN. 2006 May;17(5):1305–15.

28. Tsujikawa H, Kurotaki Y, Fujimori T, Fukuda K, Nabeshima Y-I. Klotho, a gene related to a syndrome resembling human premature aging, functions in a negative regulatory circuit of vitamin D endocrine system. Mol Endocrinol Baltim Md. 2003 Dec;17(12):2393–403.

29. Forster RE, Jurutka PW, Hsieh J-C, Haussler CA, Lowmiller CL, Kaneko I, et al. Vitamin D receptor controls expression of the anti-aging klotho gene in mouse and human renal cells. Biochem Biophys Res Commun. 2011 Oct 28;414(3):557–62.

30. Shimizu H, Bolati D, Adijiang A, Adelibieke Y, Muteliefu G, Enomoto A, et al. Indoxyl sulfate downregulates renal expression of Klotho through production of ROS and activation of nuclear factor-κB. Am J Nephrol. 2011;33(4):319–24.

31. Mitani H, Ishizaka N, Aizawa T, Ohno M, Usui S, Suzuki T, et al. In vivo klotho gene transfer ameliorates angiotensin II-induced renal damage. Hypertens Dallas Tex 1979. 2002 Apr;39(4):838–43.

32. Nakano-Kurimoto R, Ikeda K, Uraoka M, Nakagawa Y, Yutaka K, Koide M, et al. Replicative senescence of vascular smooth muscle cells enhances the calcification through initiating the osteoblastic transition. Am J Physiol Heart Circ Physiol. 2009 Nov;297(5):H1673-1684.

33. Zeisberg M, Hanai J, Sugimoto H, Mammoto T, Charytan D, Strutz F, et al. BMP-7 counteracts TGF-beta1-induced epithelial-to-mesenchymal transition and reverses chronic renal injury. Nat Med. 2003 Jul;9(7):964–8.

34. Doi S, Zou Y, Togao O, Pastor JV, John GB, Wang L, et al. Klotho inhibits transforming growth factor-beta1 (TGF-beta1) signaling and suppresses renal fibrosis and cancer metastasis in mice. J Biol Chem. 2011 Mar 11;286(10):8655–65.

35. Kendrick J, Chonchol MB. Nontraditional risk factors for cardiovascular disease in patients with chronic kidney disease. Nat Clin Pract Nephrol. 2008 Dec;4(12):672–81.

36. Phosphate and Vascular Calcification: Emerging Role of the Sodium-Dependent Phosphate Cotransporter PiT-1 [Internet]. [cited 2018 Sep 26]. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3034400/

37. Shroff RC, McNair R, Skepper JN, Figg N, Schurgers LJ, Deanfield J, et al. Chronic mineral dysregulation promotes vascular smooth muscle cell adaptation and extracellular matrix calcification. J Am Soc Nephrol JASN. 2010 Jan;21(1):103–12.

38. Faul C, Amaral AP, Oskouei B, Hu M-C, Sloan A, Isakova T, et al. FGF23 induces left ventricular hypertrophy. J Clin Invest. 2011 Nov;121(11):4393–408.

39. Komaba H, Goto S, Fujii H, Hamada Y, Kobayashi A, Shibuya K, et al. Depressed expression of Klotho and FGF receptor 1 in hyperplastic parathyroid glands from uremic patients. Kidney Int. 2010 Feb;77(3):232–8.

40. Canalejo R, Canalejo A, Martinez-Moreno JM, Rodriguez-Ortiz ME, Estepa JC, Mendoza FJ, et al. FGF23 Fails to Inhibit Uremic Parathyroid Glands. J Am Soc Nephrol JASN. 2010 Jul;21(7):1125–35.

41. Yamashita T, Yoshioka M, Itoh N. Identification of a novel fibroblast growth factor, FGF-23, preferentially expressed in the ventrolateral thalamic nucleus of the brain. Biochem Biophys Res Commun. 2000 Oct 22;277(2):494–8.

42. Shimada T, Muto T, Urakawa I, Yoneya T, Yamazaki Y, Okawa K, et al. Mutant FGF-23 responsible for autosomal dominant hypophosphatemic rickets is resistant to proteolytic cleavage and causes hypophosphatemia in vivo. Endocrinology. 2002 Aug;143(8):3179–82.

43. Kato K, Jeanneau C, Tarp MA, Benet-Pagès A, Lorenz-Depiereux B, Bennett EP, et al. Polypeptide GalNAc-transferase T3 and familial tumoral calcinosis. Secretion of fibroblast growth factor 23 requires O-glycosylation. J Biol Chem. 2006 Jul 7;281(27):18370–7.

44. Mohammadi M, Olsen SK, Ibrahimi OA. Structural basis for fibroblast growth factor receptor activation. Cytokine Growth Factor Rev. 2005 Apr;16(2):107–37.

45. Powers CJ, McLeskey SW, Wellstein A. Fibroblast growth factors, their receptors and signaling. Endocr Relat Cancer. 2000 Sep;7(3):165–97.

46. Yu X, White KE. Fibroblast growth factor 23 and its receptors. Ther Apher Dial Off Peer-Rev J Int Soc Apher Jpn Soc Apher Jpn Soc Dial Ther. 2005 Aug;9(4):308–12.

47. Yamazaki Y, Tamada T, Kasai N, Urakawa I, Aono Y, Hasegawa H, et al. Anti-FGF23 neutralizing antibodies show the physiological role and structural features of FGF23. J Bone Miner Res Off J Am Soc Bone Miner Res. 2008 Sep;23(9):1509–18.

48. Gattineni J, Bates C, Twombley K, Dwarakanath V, Robinson ML, Goetz R, et al. FGF23 decreases renal NaPi-2a and NaPi-2c expression and induces hypophosphatemia in vivo predominantly via FGF receptor 1. Am J Physiol Renal Physiol. 2009 Aug;297(2):F282-291.

49. Farrow EG, Davis SI, Summers LJ, White KE. Initial FGF23-mediated signaling occurs in the distal convoluted tubule. J Am Soc Nephrol JASN. 2009 May;20(5):955–60.

50. Shimada T, Hasegawa H, Yamazaki Y, Muto T, Hino R, Takeuchi Y, et al. FGF-23 is a potent regulator of vitamin D metabolism and phosphate homeostasis. J Bone Miner Res Off J Am Soc Bone Miner Res. 2004 Mar;19(3):429–35.

51. Weber TJ, Liu S, Indridason OS, Quarles LD. Serum FGF23 levels in normal and disordered phosphorus homeostasis. J Bone Miner Res Off J Am Soc Bone Miner Res. 2003 Jul;18(7):1227–34.

52. Wolf M. Forging forward with 10 burning questions on FGF23 in kidney disease. J Am Soc Nephrol JASN. 2010 Sep;21(9):1427–35.

53. Liu S. Fibroblast Growth Factor 23 Is a Counter-Regulatory Phosphaturic Hormone for Vitamin D. J Am Soc Nephrol. 2006 May 1;17(5):1305–15.

54. Gutiérrez OM, Mannstadt M, Isakova T, Rauh-Hain JA, Tamez H, Shah A, et al. Fibroblast growth factor 23 and mortality among patients undergoing hemodialysis. N Engl J Med. 2008 Aug 7;359(6):584–92.

55. Wolf M, Molnar MZ, Amaral AP, Czira ME, Rudas A, Ujszaszi A, et al. Elevated fibroblast growth factor 23 is a risk factor for kidney transplant loss and mortality. J Am Soc Nephrol JASN. 2011 May;22(5):956–66.

56. Jean G, Terrat J-C, Vanel T, Hurot J-M, Lorriaux C, Mayor B, et al. High levels of serum fibroblast growth factor (FGF)-23 are associated with increased mortality in

long haemodialysis patients. Nephrol Dial Transplant Off Publ Eur Dial Transpl Assoc - Eur Ren Assoc. 2009 Sep;24(9):2792–6.

57. Seiler S, Reichart B, Roth D, Seibert E, Fliser D, Heine GH. FGF-23 and future cardiovascular events in patients with chronic kidney disease before initiation of dialysis treatment. Nephrol Dial Transplant Off Publ Eur Dial Transpl Assoc - Eur Ren Assoc. 2010 Dec;25(12):3983–9.

58. Ferrari SL, Bonjour J-P, Rizzoli R. Fibroblast growth factor-23 relationship to dietary phosphate and renal phosphate handling in healthy young men. J Clin Endocrinol Metab. 2005 Mar;90(3):1519–24.

59. Isakova T, Gutiérrez OM, Smith K, Epstein M, Keating LK, Jüppner H, et al. Pilot study of dietary phosphorus restriction and phosphorus binders to target fibroblast growth factor 23 in patients with chronic kidney disease. Nephrol Dial Transplant Off Publ Eur Dial Transpl Assoc - Eur Ren Assoc. 2011 Feb;26(2):584–91.

60. Gonzalez-Parra E, Gonzalez-Casaus ML, Galán A, Martinez-Calero A, Navas V, Rodriguez M, et al. Lanthanum carbonate reduces FGF23 in chronic kidney disease Stage 3 patients. Nephrol Dial Transplant Off Publ Eur Dial Transpl Assoc - Eur Ren Assoc. 2011 Aug;26(8):2567–71.

61. Koiwa F, Kazama JJ, Tokumoto A, Onoda N, Kato H, Okada T, et al. Sevelamer hydrochloride and calcium bicarbonate reduce serum fibroblast growth factor 23 levels in dialysis patients. Ther Apher Dial Off Peer-Rev J Int Soc Apher Jpn Soc Apher Jpn Soc Dial Ther. 2005 Aug;9(4):336–9.

62. Guo Y-C, Yuan Q. Fibroblast growth factor 23 and bone mineralisation. Int J Oral Sci. 2015 Mar;7(1):8–13.

63. Erben RG. Physiological Actions of Fibroblast Growth Factor-23. Front Endocrinol [Internet]. 2018 May 28 [cited 2018 Sep 9];9. Available from: https://www.frontiersin.org/article/10.3389/fendo.2018.00267/full

64. Olauson H, Lindberg K, Amin R, Sato T, Jia T, Goetz R, et al. Parathyroid-specific deletion of Klotho unravels a novel calcineurin-dependent FGF23 signaling pathway that regulates PTH secretion. PLoS Genet. 2013;9(12):e1003975.

65. Galitzer H, Ben-Dov IZ, Silver J, Naveh-Many T. Parathyroid cell resistance to fibroblast growth factor 23 in secondary hyperparathyroidism of chronic kidney disease. Kidney Int. 2010 Feb;77(3):211–8.

66. Evenepoel P, Wolf M. A balanced view of calcium and phosphate homeostasis in chronic kidney disease. Kidney Int. 2013 May 1;83(5):789–91.

67. Prasad N, Bhadauria D. Renal phosphate handling: Physiology. Indian J Endocrinol Metab. 2013;17(4):620–7.

68. Isakova T, Wahl P, Vargas GS, Gutiérrez OM, Scialla J, Xie H, et al. Fibroblast growth factor 23 is elevated before parathyroid hormone and phosphate in chronic kidney disease. Kidney Int. 2011 Jun;79(12):1370–8.

69. Hill Gallant KM, Spiegel DM. Calcium Balance in Chronic Kidney Disease. Curr Osteoporos Rep. 2017;15(3):214–21.

70. Cannata-Andía JB, Carrera F. The Pathophysiology of Secondary Hyperparathyroidism and the Consequences of Uncontrolled Mineral Metabolism in Chronic Kidney Disease: The Role of COSMOS. NDT Plus. 2008 Jan;1(Suppl 1):i2–6.

71. FGF23, PTH and Phosphorus Metabolism in the Chronic Renal Insufficiency Cohort [Internet]. [cited 2018 Sep 28]. Available from: https://www.ncbi.nlm.nih.gov/ pmc/articles/PMC3134393/

72. Ketteler M. Phosphate Metabolism in CKD Stages 3–5: Dietary and Pharmacological Control. Int J Nephrol [Internet]. 2011 May 23 [cited 2018 Sep 28];2011. Available from: https://www.ncbi.nlm.nih.gov/ pmc/articles/ PMC3108253/

73. Serum phosphate levels and mortality risk among people with chronic kidney disease. - PubMed - NCBI [Internet]. [cited 2018 Sep 28]. Available from: https://www.ncbi.nlm.nih.gov/ pubmed/15615819

74. Hasegawa H, Nagano N, Urakawa I, Yamazaki Y, Iijima K, Fujita T, et al. Direct evidence for a causative role of FGF23 in the abnormal renal phosphate handling

and vitamin D metabolism in rats with early-stage chronic kidney disease. Kidney Int. 2010 Nov;78(10):975–80.

75. Slatopolsky E, Bricker NS. The role of phosphorus restriction in the prevention of secondary hyperparathyroidism in chronic renal disease. Kidney Int. 1973 Aug 1;4(2):141–5.

76. Craver L, Marco MP, Martinez I, Rue M, Borras M, Martin ML, et al. Mineral metabolism parameters throughout chronic kidney disease stages 1-5--achievement of K/DOQI target ranges. Nephrol Dial Transplant. 2007 Jan 25;22(4):1171–6.

77. Pavik I, Jaeger P, Ebner L, Wagner CA, Petzold K, Spichtig D, et al. Secreted Klotho and FGF23 in chronic kidney disease Stage 1 to 5: a sequence suggested from a cross-sectional study. Nephrol Dial Transplant. 2013 Feb 1;28(2):352–9.

78. Tartaglione L, Pasquali M, Rotondi S, Muci ML, Leonangeli C, Farcomeni A, et al. Interactions of sclerostin with FGF23, soluble klotho and vitamin D in renal transplantation. PLoS ONE [Internet]. 2017 May 30 [cited 2018 Sep 28];12(5). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5448809/

79. Sakan H, Nakatani K, Asai O, Imura A, Tanaka T, Yoshimoto S, et al. Reduced Renal a-Klotho Expression in CKD Patients and Its Effect on Renal Phosphate Handling and Vitamin D Metabolism. PLOS ONE. 2014;9(1):9.

Annexures

INSTITUTIONAL ETHICS COMMITTEE
MADRAS MEDICAL COLLEGE, CHENNAI 600 003

EC Reg.No.ECR/270/Inst./TN/2013
Telephone No.044 25305301
Fax: 011 25363970

CERTIFICATE OF APPROVAL

To
Dr.D.Kalpana
I Year PG in MD Bio-Chemistry
Institute of Bio-Chemistry
Madras Medical College
Chennai 600 003

Dear Dr.D.Kalpana,

The Institutional Ethics Committee has considered your request and approved your study titled **"SERUM SOLUBLE α KLOTHO AND FGF 23 LEVELS IN CHRONIC KIDNEY DISEASE " - NO.14032017(I)**

The following members of Ethics Committee were present in the meeting hold on **02.03.2017** conducted at Madras Medical College, Chennai 3

1.Dr.C.Rajendran, MD.,	:Chairperson
2.Dr. K.Narayanasamy,MD,DM.,Dean(FAC), MMC,Ch-3	:Deputy Chairperson
3.Prof.Sudha Seshayyan,MD., Vice Principal,MMC,Ch-3	: Member Secretary
4.Prof.S.Suresh, MS, Prof. of Surgery,MMC,Ch-3	: Member
5.Prof.Baby Vasumathi,MD.,Director, Inst. of O & G	: Member
6.Prof.K.Ramadevi,MD.,Director,Inst.of Bio-Che,MMC,Ch-3	: Member
7.Prof.R.Padmavathy, MD, Director,Inst.of Pathology,MMC,Ch-3	: Member
8.Tmt.J.Rajalakshmi, JAO,MMC, Ch-3	: Lay Person
9.Thiru S.Govindasamy, BA.,BL,High Court,Chennai	: Lawyer
10.Tmt.Arnold Saulina, MA.,MSW.,	:Social Scientist

We approve the proposal to be conducted in its presented form.

The Institutional Ethics Committee expects to be informed about the progress of the study and SAE occurring in the course of the study, any changes in the protocol and patients information/informed consent and asks to be provided a copy of the final report.

15/6/17

Member Secretary – Ethics Committee

MEMBER SECRETARY
INSTITUTIONAL ETHICS COMMITTEE
MADRAS MEDICAL COLLEGE
CHENNAI-600 003

URKUND

Urkund Analysis Result

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Submitted By: drdkalpana84@gmail.com
Significance: 11 %

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https://www.hindawi.com/journals/ije/2015/872193/
http://insanemedicine.com/wp-content/uploads/2016/06/New-insights-into-the-FGF23-Klotho-axis..pdf
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3983685/
https://www.pubfacts.com/detail/30159331/Correlation-between-Soluble-Klotho-and-Renal-Function-in-Patients-with-Chronic-Kidney-Disease-A-Revi
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https://www.hindawi.com/journals/ijn/2011/364890/
https://journals.lww.com/co-nephrolhypertens/Pages/ArticleViewer.aspx?year=2018&issue=07000&article=00003&type=Fulltext

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PLAGIARISM CERTIFICATE

This is to certify that this dissertation work titled **"SERUM SOLUBLE α KLOTHO AND FGF 23 LEVELS IN CHRONIC KIDNEY DISEASE"** of the candidate **DR. D. KALPANA** with registration Number **201623003** for the award of **M.D** in the branch of **BIOCHEMISTRY**. I personally verified the urkund.com website for the purpose of plagiarism Check. I found that the uploaded thesis file contains from introduction to conclusion pages and result shows **11 percentage** of plagiarism in the dissertation.

Guide & Supervisor sign with Seal.

PROFORMA

Name :

Age/sex:

Height :

Weight :

BMI :

IP/OP No:

Ward:

History:

Examination :

Investigations:

1. Serum Urea
2. Serum Creatinine
3. Serum Calcium
4. Serum Phosphorus
5. Serum FGF 23
6. Serum soluable alpha klotho
7. Serum PTH
8. Serum Vitamin D
9. Spot Urine PCR
10. eGFR

INFORMATION SHEET

- Your valuable blood sample has been accepted.
- We are conducting a study on serum soluble α Klotho and FGF 23 levels on CKD patients at Rajiv Gandhi Government General Hospital, Chennai.
- The purpose of this study is to correlate the levels of soluble α Klotho and FGF 23 levels in CKD patients and their relationship with CKD-MBD with the help of certain special tests.
- We are selecting certain cases, and if your blood sample is found eligible, we may be using your blood sample to perform extra tests and special studies, which in any way, do not affect your final report or management.
- The privacy of the patients in the research will be maintained throughout the study. In the event of any publication or presentation resulting from the research, no personally identifiable information will be shared.
- Taking part in this study is voluntary. You are free to decide whether to participate in this study or to withdraw at any time; your decision will not result in any loss of benefits, to which you are otherwise entitled.
- The results of the special study may be intimated to you, at the end of the study period or during the study, if anything is found abnormal, which may aid in the management or treatment.

Signature of investigator Signature of participant

Date:

PATIENT CONSENT FORM

Title of the study **SERUM SOLUBLE α KLOTHO AND FGF 23 LEVELS IN CHRONIC KIDNEY DISEASE**

Name : Date :

Age : OP No :

Sex : Project Patient No :

The details of the study have been provided to me in writing and explained to me in my own language.

I confirm that I have understood the above study and had the opportunity to ask questions.

I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason, without the medical care that will normally be provided by the hospital being affected.

I agree to use my personal clinical history & investigation details for the purpose of the study.

I agree not to restrict the use of any data or results that arise from this study provided such a use is only for scientific purpose(s).

I have been given an information sheet giving details of the study.

Having understood _____s/o_____ give my consent to participate in the study conducted by DR D.KALPANA, Post graduate, Institute of Biochemistry, Madras Medical College, Chennai.

Signature of the investigator:

Signature of the participant

Thumb impression

Chennai

Date:

ஆராய்ச்சி தகவல் தாள்

தங்களது இரத்தம் இங்குபெற்றுக்கொள்ளப்பட்டது.

சென்னை அரசுபொது மருத்துவமனையில் ''நாட்பட்ட சிறுநீரக கோளாறுகளில் இரத்தத்தில் ஆல்ஃபா க்ளோதோ மற்றும் எப்.ஜி.எப். 23 அளவு பற்றிய ஆய்வு" என்ற தலைப்பில் ஆராய்ச்சி நடைபெற்று வருகின்றது.

நீங்களும் இந்த ஆராய்ச்சியில் பங்கேற்க நாங்கள் விரும்பிகிறோம்.

இந்த ஆராய்ச்சியில் உங்களுடைய இரத்தம் எடுத்து சிறப்புப்பரி சோதனைக்கு உட்படுத்தி அதன் தகவல்களை ஆராய்வோம்.

அதனால் தங்களது நோயின் ஆய்வறிக்கையோ அல்லது சிகிச்சையோ பாதிப்புக்கு உள்ளாகாது என்பதையும் தெரிவித்துக் கொள்கிறோம்.

இந்த ஆய்வில் பங்கு பெறுபவர்கள் இரண்டு பிரிவுகளாக பிரிக்கப்படுவார்கள். பிரிவு-A : நாள்பட்ட சிறுநீரக கோளாறு உடையவர்கள். பிரிவு-B : ஆரோக்கியமானவர்கள்.

முடிவுகளை அல்லது கருத்துக்களை வெளியிடும் போதோ அல்லது ஆராய்ச்சியின் போதோ தங்களது பெயரையோ அல்லது அடையாளங்களையோ வெளியிடமாட்டோம் என்பதையும் தெரிவித்துக்கொள்கிறோம்

இந்த ஆராய்ச்சியில் பங்கேற்பது தங்களுடைய விருப்பத்தின் பேரில் தான் இருக்கிறது.மேலும் நீங்கள் எந்நேரமும் இந்த ஆராய்ச்சியிலிருந்து பின்வாங்கலாம் என்பதையும் தெரிவித்துகொள்கிறோம்.

இந்த சிறப்பு பரிசோதனைகளின் முடிவுகளை ஆராய்ச்சியின் போது அல்லது ஆராய்ச்சியின் முடிவின் போது தங்களுக்கு அறிவிப்போம் என்பதையும் தெரிவித்துக்கொள்கிறோம்.

ஆராய்ச்சியாளர் கையொப்பம் பங்கேற்பாளர் கையொப்பம்

தேதி:

நோயாளியின் ஒப்புதல் படிவம்

ஆராய்ச்சி தலைப்பு: நாட்பட்ட சிறுநீரக கோளாறுகளில் இரத்தத்தில் ஆல்ஃபா க்ளோதோ மற்றும் எப்.ஜி.எப். 23 அளவு பற்றிய ஆய்வு

பெயர் : தேதி :

வயது : புறநோயாளிஎண்:

பால் : ஆராய்ச்சி சேர்க்கை எண் :

இந்த ஆராய்ச்சியின் விவரங்களும் அதன் நோக்கங்களும் முழுமையாக எனக்கு தெளிவாக விளக்கப்பட்டது.

எனக்கு விளக்கப்பட்ட விஷயங்களை புரிந்து கொண்டு நான் எனது சம்மதத்தைத் தெரிவிக்கிறேன்.

இந்த ஆராய்ச்சியில் பிறரின் நிர்பந்தமின்றி என் சொந்த விருப்பத்தின் பேரில் தான் பங்கு பெறுகிறேன் மற்றும் நான் இந்த ஆராய்ச்சியிலிருந்து எந்நேரமும் பின்வாங்கலாம் என்பதையும், அதனால் எந்த பாதிப்பும் ஏற்படாது என்பதையும் புரிந்து கொண்டேன்நான் "நாட்பட்ட சிறுநீரக கோளாறுகளில் இரத்தத்தில் ஆல்ஃபா க்ளோதோ மற்றும் எப்.ஜி.எப். 23 அளவு பற்றிய ஆய்வு" என்ற தலைப்பில் மேற்கொள்ளப்படும் இந்த ஆராய்ச்சியின் விபரங்களைக் கொண்ட தகவல் தாளைப் பெற்றுக் கொண்டேன்.

இதன் மூலம் எந்த பின்விளைவும் வராது என மருத்துவர் மூலம் தெரிந்து கொண்டு என்னுடைய சுயநினைவுடன் மற்றும் முழு சுதந்திரத்துடன் இந்த மருத்துவ ஆராய்ச்சியில் என்னை சேர்த்துக்கொள்ள சம்மதிக்கிறேன்

தேதி கையொப்பம்